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ORIGINAL RESEARCH

Causal Effects of Inflammatory Cytokines and Immune Cell Phenotypes on Spontaneous Abortion: Evidence From Mendelian Randomization

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Purpose: This study aims to investigate the causal relationship between inflammatory cytokines, immune cells and spontaneous abortion (SA).

Methods: A bidirectional two-sample Mendelian randomization (MR) analyses was conducted based on the genetic data of 91 inflammatory cytokines (n=14,824), 731 immune traits (n=3757) and SA (18,680 cases and 162,987 controls) cohorts. Five different MR analysis methods and Bayesian-weighted Mendelian randomization (BWMR) analysis were employed to assess the genetic causal connection. In addition, the robustness of this study results was ensured through comprehensive sensitivity analyses assessing heterogeneity, and potential horizontal pleiotropy and reverse causality.

Results: These MR results suggest that higher levels of two inflammatory cytokines and ten immune cells are associated with a lower risk of SA (OR < 1.00). In contrast, fifteen immune cell traits exhibit a positive relationship with SA risk (OR > 1.00). Notably, mediation analysis revealed that the causal effect of programmed death ligand 1 (PDL1) on SA was partially mediated by CD45 expression on Granulocytic Myeloid-Derived Suppressor Cells (GR-MDSCs), and Terminally Differentiated $CD4^-CD8^-$ T cells also acted as mediators in the causal effect of tumor necrosis factor-beta (TNF-β) on SA.

Conclusion: This study comprehensively assessed the causal relationship between immune-related exposures and SA, identifying several immune factors associated with SA risk. These finding have implications for clinical guidance in pregnancy preparation.

Keywords: inflammatory cytokine, immune cell, Mendelian randomization, spontaneous abortion

Introduction

Spontaneous abortion (SA), with a global incidence of approximately 15.3%, is among the most prevalent complications during the first trimester of pregnancy.[1] The etiology of SA is multifactorial, including chromosomal abnormalities, anatomical defects, infections, as well as hormonal and psychiatric disorders.[2,3] Additionally, substantial evidence suggests that dysregulation of both peripheral and maternal-fetal immune systems is associated with abnormal pregnancy outcomes.[4,5] During pregnancy, appropriate systemic or uterine inflammation is essential for the normal development of

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the pregnancy. Specialized immune interactions at the maternal-fetal interface, along with regulatory adaptations of the maternal immune system, establish maternal immune tolerance toward the fetus.[6] SA may occur with either excessive or insufficient levels of circulating inflammatory cytokines, such as leukemia inhibitory factor (LIF), interleukin-6 (IL-6), programmed death ligand 1 (PDL1), and tumor necrosis factor-alpha (TNF-α).[5,7,8] The pathophysiology of immunological SA is intricate and warrant further investigation.

The relationship between inflammatory cytokines, immune cells and SA have not been clearly identified in previous studies, despite considerable efforts to elucidate their functional roles during pregnancy. For instance, Aoki et al demonstrated that women with increased peripheral blood natural killer (pNK) cell activity had a higher relative risk of miscarriage in subsequent pregnancies compared to those with normal NK cell activity.[9] Similarly, Matsubayashi found that elevated pNK cytotoxicity and activity were significant risk factors impeding successful pregnancy.[10] Contrarily, a recent meta-analysis reported no significant differences in pNK activity between women experiencing SA and those with normal pregnancies.[11] These discrepancies may stem from the inherent complexity and functional heterogeneity of the immune system, limited sample sizes, and variability in experimental design across previous studies. More recently, a large-scale population-based retrospective cohort study suggested that pre-pregnancy peripheral blood leukocytes and their subsets are associated with SA risk.[4] Specifically, leukocytosis was found to be linked to a reduced risk of SA, whereas leukopenia was associated with an increased risk. These findings underscore the potential benefit of monitoring and optimizing leukocyte levels prior to conception as a strategy for reducing SA risk, thus providing a theoretical foundation for preconception immune interventions. However, to implement these interventions effectively, it remains imperative to clarify a well-defined causal relationship between immune factors and SA.

Mendelian Randomization (MR) analysis is a widely recognized and powerful approach for inferring causal relationships.[12] Unlike traditional case-control or observational studies, MR utilizes genetic variants as instrumental variables that are randomly allocated from parental to offspring during gametogenesis. This genetic randomization process, combined with large sample sizes, significantly reduces confounding bias. In addition, methods such as robust independence testing, pleiotropy assessments, and multiple sensitivity analyses further reinforcing the robustness and credibility of causal inference.[13] For example, a recent MR study found no evidence of a causal link between vitamin D levels and miscarriage,[14] demonstrating the utility of MR in deriving meaningful and clinically relevant conclusions.

In this study, we comprehensively evaluated the causal relationships between 91 peripheral blood inflammatory cytokines and 731 immune cell traits with the risk of SA, and investigated whether immune cell signatures could mediate this association. A comprehensive evaluation of their links will facilitate a deeper understanding of the immunopathogenesis of SA. Overall, this study provides a genetic perspective on the relationship between immunity and miscarriage, which holds implications for mitigation of the risk of SA.

Materials and Methods

Study Design

The overall study design is illustrated in Figure 1. Firstly, two-sample bidirectional MR analysis were conducted to investigate the causal relationships between inflammatory cytokines, immune cells and SA. Subsequently, we assessed the causal impact of inflammatory cytokines on immune cells and calculated the mediation proportion of each mediator. Finally, pathway and functional analyses were performed to elucidate the potential mechanisms through which inflammatory cytokines and immune cells may contribute to the pathogenesis of SA.

Data Sources

The genome-wide association study (GWAS) summary statistics for 91 inflammatory cytokines were sourced from Zhao et al, which integrated the plasma proteomics data and genome-wide protein quantitative trait locus (pQTL) data utilizing the Olink Target platform.[15] This platform is capable of detecting low-abundance immune-related cytokines in peripheral blood plasma with high specificity and sensitivity. This study encompassed 14,824 predominantly European participants across 11 cohorts. The per-protein GWAS data is publicly accessible in the GWAS Catalog (https://www.ebi.ac.uk/gwas/, accession numbers GCST90274758 to GCST90274848). Additionally, the GWAS summary statistics for immune cells

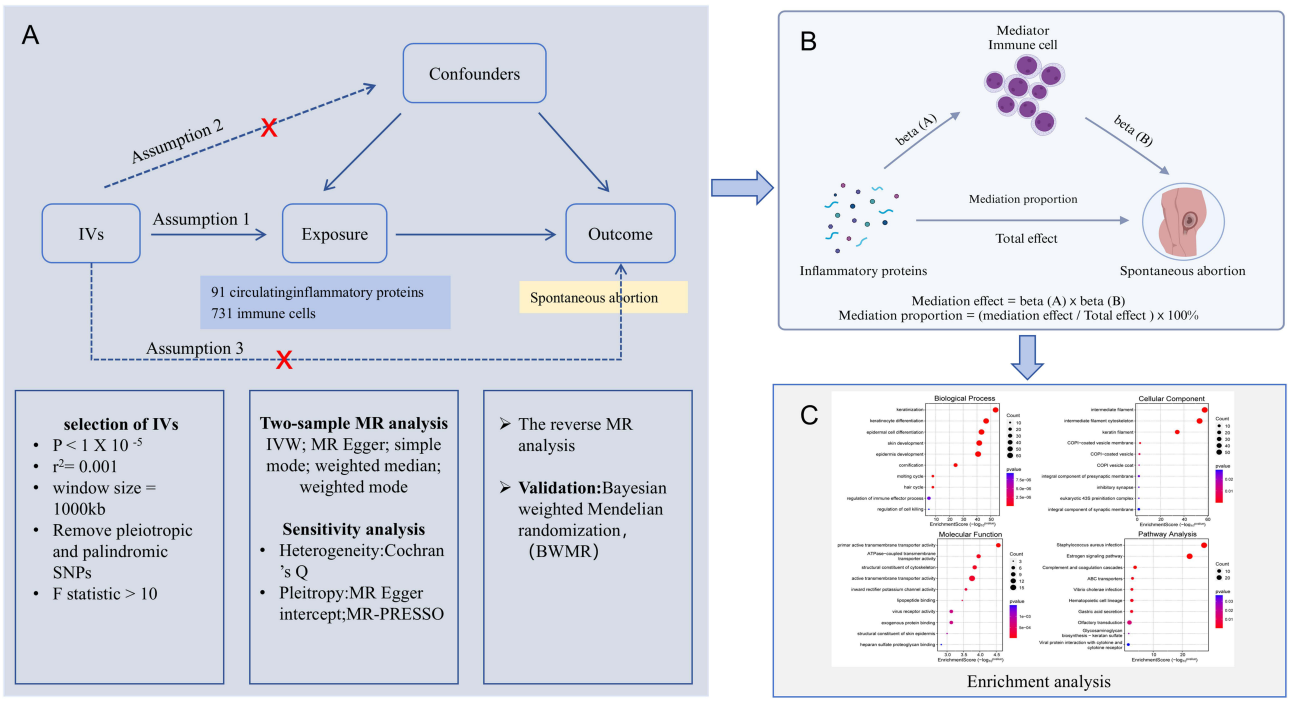

Figure 1 Overview of study design of the bidirectional Mendelian randomization framework used to investigate the causal effect of inflammatory cytokines and immune cell subtypes on spontaneous abortion. (**A**) Bidirectional Mendelian randomization. (**B**) Mediation Mendelian randomization. (**C**) Enrichment analysis.
Abbreviation: SNPs, single-nucleotide polymorphisms.

were obtained from the GWAS Catalog under the study accession numbers GCST90001391 to GCST90002121. In this study, Orrù et al conducted a GWAS analysis on approximately 22 million single nucleotide polymorphisms (SNPs) across 731 immune cell traits in a cohort of 3757 Sardinians.[16] Peripheral blood immune cell traits were quantified using high-throughput flow cytometry, enabling a comprehensive analysis of immune phenotypes in circulation. The 731 immune cell traits encompassed six immunophenotypes, including median fluorescence intensities (MFI) reflecting surface antigen levels (n=389), relative cell (RC) counts (n=192), absolute cell (AC) counts (n=118), and morphological parameters (MP) (n=32). These immune cells were further classified into seven panels: T cells maturation stage, TBNK (T cells, B cells, and natural killer cells), Treg, B cells, classical dendritic cells (CDC), monocytes, and myeloid cells.

The GWAS summary data for SA was derived from the FinnGen (https://www.finngen.fi/fi.finngen_R10_O15_ABORT_SPONTAN) involving 18,680 cases and 162,987 controls of European ancestry to identify risk loci related to SA.[17] SA refers to the natural death of an embryo or fetus before it can survive independently.[18] The diagnosis of SA was coded according to the International Classification of Diseases (ICD) as follows: ICD-10O03, ICD-9634, and ICD-8643. The control group consists of female individuals who have not experienced any form of pregnancy loss, including ectopic pregnancy, hydatidiform mole, SA, medical abortion, unspecified abortion, failed abortion attempts, complications following abortion, abnormal products of conception, or other types of abortion. All the participants in this present study were from Europe, with no overlapping cohorts.

All data used in this study were derived from publicly available databases, where ethical approval and informed consent were previously obtained in the original studies. According to Article 16 of the Measures for the Ethical Review of Life Sciences and Medical Research Involving Humans in China, which allows exemption from ethical review for research based on legally obtained public or anonymized data, no additional ethical approval was necessary for this study.

Selection of Instrumental Variables

To predict the causal effect, instrumental variables (IVs) must meet three fundamental assumptions:[19] (1) the relevance assumption, which requires that IVs exhibit a strong association with the exposure; (2) the independence assumption,

which mandates that IVs are not related to any confounding factors; and (3) the exclusionary assumption, which stipulates that IVs influence the outcome exclusively through the exposure and not through any other pathway.

Following previous research, SNPs with a p-value less than 1e×10-5 were selected as IVs significantly associated with inflammatory proteins and immune cells.[20,21] SNPs used as IVs for SA were subject to a stricter criterion ($p < 5e\times10\text{-}6$). To ensure the included SNPs were mutually independent and to eliminate the genetic pleiotropy, we utilized PLINK software to prune these SNPs with linkage disequilibrium (LD) coefficient $r^2 > 0.001$ and physical distance < 10,000 kb.[22] The statistical strength of IVs for each exposed trait was assessed using F-statistics, with weak SNPs (F-statistics < 10) being excluded to avoid associations with unmeasured confounders.[23] Furthermore, we employed the LDLink-LDTrait approach to exclude SNPs related to confounding factors such as body mass index, body weight, smoking, alcohol consumption, and nicotine intake.[18] Finally, palindromic and incompatible SNPs were removed during the harmonization of IVs with the outcome. These steps ensure a robust selection of IVs, critical for reliable causal inference in MR studies.

Pathway and Functional Enrichment Analyses

Functional Mapping and Annotation of Genome-Wide Association Studies (FUMA, https://fuma.ctglab.nl/snp2gene) was used for functional annotating.[24] We employed three distinct mapping strategies within the SNP2GENE module of FUMA: positional mapping, eQTL-based gene mapping, and gene mapping based on three-dimensional chromatin interaction, to identify genes closely associated with the selected IVs for MR analysis. Subsequently, we conducted pathway and functional enrichment analyses and visualizations on the online platform (https://www.bioinformatics.com.cn).[25] The Gene Ontology (GO) analysis provided gene function annotations at three levels: cellular components (CC), molecular functions (MF), and biological processes (BP). Meanwhile, the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis detailed the signaling pathways enriched among these genes.[26]

Mendelian Randomization Analysis

All statistical analyses were conducted using the "TwoSampleMR", "MRPRESSO" and "BWMR" packages within R software (version 4.2.0). To predict the causal associations, we employed five common MR approaches: inverse variance weighting (IVW), MR-Egger, weighted median, simple mode, and weighted mode.[27] Among these, IVW was designated as the primary analytical method due to its accuracy in the absence of heterogeneity and the presence of horizontal pleiotropy.[28] The results from the other four methods served as complementary evidence to those results obtained via IVW. Subsequently, a series of rigorous sensitivity analyses were undertaken to ensure the robustness of the MR analysis results. The degree of heterogeneity was assessed using Cochran's Q statistic based on the IVW and MR-Egger methods. To detect horizontal pleiotropy, we applied the MR-Egger regression intercept and the MR-PRESSO global test. Additionally, leave-one-out analysis was conducted to determine if the causal effect was significantly influenced by any single SNP. Bayesian-weighted Mendelian randomization (BWMR) analysis, a novel and robust tool for inferring causality, was employed to further validate the causal relationship identified by IVW. This method has proven effective in reducing the occurrence of false-positive results, offering significant advantages in both accuracy and statistical efficacy.[29] *P*-value < 0.05 was considered to have a significant association between exposure and outcome. Finally, reverse MR analysis was conducted to investigate whether SA had a causal effect on the identified inflammatory proteins and immune cells. In this case, SNPs associated with SA were selected as IVs because SA was regarded as exposure. The reverse MR analysis procedure was similar to that used for the MR analysis.

Results

Causal Effects of Inflammatory Cytokines on SA

Detailed information on the two-sample MR analysis of 91 inflammatory cytokines on SA was demonstrated in Supplementary Table S1. Based on the IVW method, our results showed that an elevated level of PDL1 was associated with a reduced risk of SA (odds ratio (OR) = 0.920, 95% confidence interval (CI) [0.858, 0.986], p = 0.019) (Figure 2). Additionally, there was a significant association between tumor necrosis factor-beta (TNF-β) and SA, where higher TNF-

β levels implied a lower SA risk (OR = 0.965, 95% CI [0.935,0.996], p = 0.026). No heterogeneity or horizontal pleiotropy was identified by Cochran's Q statistic, MR-Egger intercept test, and MR-PRESSO in this MR analysis (Figure 2). These findings were further corroborated using BWMR, confirming the negative effect for PDL1 (OR = 0.917, 95% CI [0.853, 0.986], p = 0.019) and TNF-β (OR = 0.964, 95% CI [0.933, 0.995], p = 0.025) in reducing SA risk (Figure 3). Reverse MR analysis suggested that SA could not influence the level of PDL1 and TNF-β, indicating a unidirectional causal relationship (Supplementary Table S2).

Causal Effects of Immune Cells on SA

Subsequently, a two-sample MR analysis was performed to explore the causal relationship between 731 immune cell traits and SA (Supplementary Table S3). By integrating results from both MR-IVW and BWMR analyses, we identified 25 immune cell traits significantly associated with SA risk (Figures 4 and 5). Among these, 10 immune cell traits appeared to function as protective factors against SA. Elevated levels of CD38 on CD20− B cell, CD25 on IgD− CD38dim B cell, CD127 on CD8+ T cell, CD3 on Natural Killer T (NKT) cell, CD45 on Granulocytic Myeloid-Derived Suppressor Cells (GR-MDSCs), CD80 on granulocyte, CD4+ T cells, CCR2 on CD14+ CD16+ monocyte, CD28+ CD45RA+ CD8+ T cell (AC) and CD28+ CD45RA+ CD8+ T cell (RC) was inversely associated with the risk of SA. These associations were robust, showing no evidence of heterogeneity, horizontal pleiotropy, or reverse causality (Supplementary Tables S4 and S5).

Conversely, 15 immune cell traits were identified as risk factors for SA. The increased level of IgD+ CD38dim B cell, CD28− CD8dim T cell, CD8 on HLA DR+ CD8+ T cell, CD24 on IgD+ CD38- B cell, CD28 on activated CD4 regulatory T cell, CD3 on Effector Memory CD4+ T cell, Terminally Differentiated CD4− CD8− T cell, CD28− CD4−

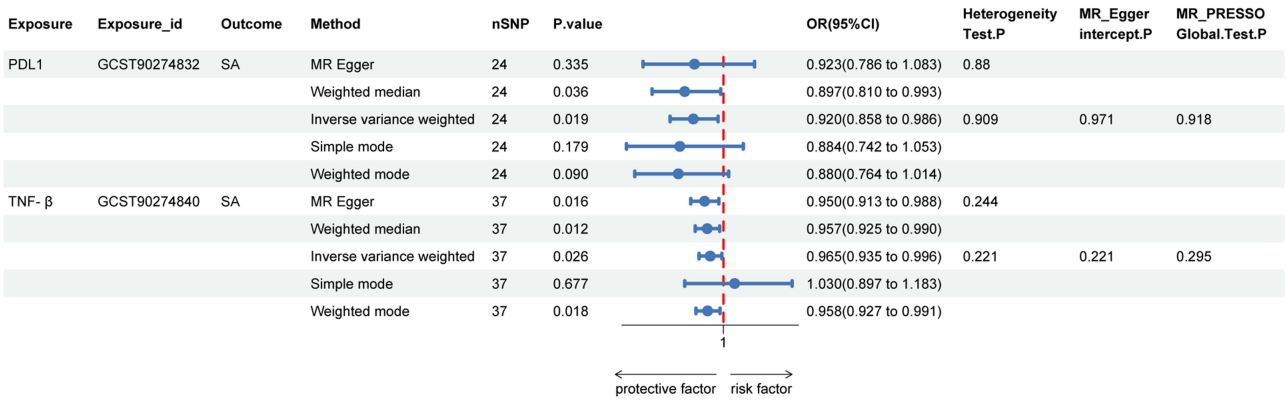

Figure 2 Mendelian randomization analysis showed the causality of 2 inflammatory cytokines on SA was essential.
Abbreviations: PDL1, Programmed cell death 1 ligand 1; TNF-β, Tumor necrosis factor – beta; SA, Spontaneous abortion; CI, confidence interval; SNPs, single-nucleotide polymorphisms.

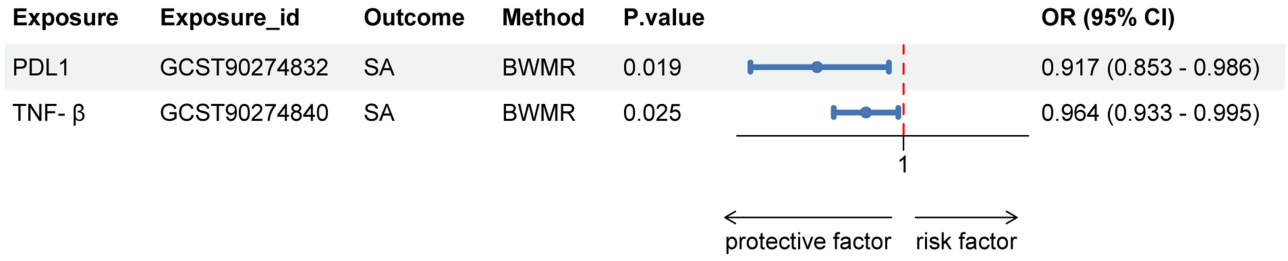

Figure 3 Bayesian-weighted Mendelian randomization analysis validates the causal relationship between SA and the 2 inflammatory cytokines.
Abbreviations: PDL1, Programmed cell death 1 ligand 1; TNF-β, Tumor necrosis factor – beta; SA, Spontaneous abortion; CI, confidence interval; BWMR, Bayesian-weighted Mendelian randomization.

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Exposure	Exposure_id	Outcome	Method	nSNP	P.value	OR(95%CI)	Heterogeneity Test.P	MR_Egger intercept.P	MR_PRESSO Global.Test.P
CD38 on CD20- B cell	GCST90001809	SA	IVW	19	0.029	0.945(0.898 to 0.994)	0.215	0.092	0.261
CD25 on IgD- CD38dim B cell	GCST90001789	SA	IVW	20	0.001	0.949(0.920 to 0.978)	0.884	0.751	0.905
CD127 on CD8+ T cell	GCST90001927	SA	IVW	24	0.007	0.958(0.928 to 0.989)	0.842	0.476	0.844
CD3 on Natural Killer Tcell	GCST90001848	SA	IVW	17	0.020	0.965(0.936 to 0.994)	0.964	0.735	0.97
CD45 on GR− MDSC	GCST90002047	SA	IVW	18	0.021	0.967(0.940 to 0.995)	0.141	0.08	0.154
CD80 on granulocyte	GCST90002040	SA	IVW	31	0.003	0.967(0.946 to 0.989)	0.307	0.374	0.321
CD4+ T cell	GCST90001590	SA	IVW	22	0.028	0.973(0.950 to 0.997)	0.604	0.705	0.675
CCR2 on CD14+ CD16+ monocyte	GCST90001992	SA	IVW	37	0.004	0.976(0.959 to 0.992)	0.01	0.861	0.019
CD28+ CD45RA+ CD8+ T cell(AC)	GCST90001690	SA	IVW	54	0.031	0.995(0.991 to 0.999)	0.898	0.338	0.895
CD28+ CD45RA+ CD8+ Tcell(RC)	GCST90001688	SA	IVW	88	0.013	0.996(0.994 to 0.999)	0.958	0.88	0.963
IgD+ CD38dim B cell	GCST90001394	SA	IVW	25	0.046	1.013(1.001 to 1.026)	0.678	0.974	0.685
CD28- CD8dim T cell	GCST90001662	SA	IVW	15	0.010	1.013(1.003 to 1.023)	0.463	0.154	0.604
CD8 on HLA DR+ CD8+ T cell	GCST90002060	SA	IVW	25	0.034	1.020(1.002 to 1.038)	0.81	0.97	0.852
CD24 on IgD+ CD38- B cell	GCST90001766	SA	IVW	21	0.022	1.024(1.003 to 1.044)	0.987	0.964	0.987
CD28 on activated CD4 regulatory T cell	GCST90001902	SA	IVW	18	0.037	1.027(1.002 to 1.053)	0.752	0.366	0.734
CD3 on Effector Memory CD4+ T cell	GCST90001843	SA	IVW	19	0.024	1.027(1.004 to 1.051)	0.742	0.518	0.791
Terminally Differentiated CD4- CD8- T cell	GCST90001573	SA	IVW	24	0.029	1.028(1.003 to 1.054)	0.474	0.718	0.412
CD28- CD4- CD8- T cell(RC)	GCST90001694	SA	IVW	22	0.028	1.032(1.003 to 1.061)	0.685	0.558	0.735
Granulocyte TBNK	GCST90001652	SA	IVW	25	0.017	1.032(1.006 to 1.059)	0.589	0.177	0.625
CD28− CD127− CD25++ CD8+ T cell	GCST90001673	SA	IVW	18	0.025	1.035(1.004 to 1.067)	0.147	0.792	0.316
Central Memory CD4+ T cell	GCST90001539	SA	IVW	24	0.008	1.039(1.010 to 1.069)	0.831	0.807	0.859
CD25 on transitional B cell	GCST90001795	SA	IVW	22	0.003	1.048(1.016 to 1.081)	0.557	0.662	0.593
CD25++ CD8+ T cell	GCST90001679	SA	IVW	17	0.016	1.050(1.009 to 1.093)	0.213	0.51	0.266
CD25 on IgD+ CD38+ B cell	GCST90001783	SA	IVW	13	0.012	1.055(1.012 to 1.100)	0.379	0.393	0.453
IgD+ CD24+ B cell	GCST90001439	SA	IVW	15	0.023	1.058(1.008 to 1.110)	0.393	0.58	0.422

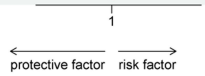

Figure 4 MR analysis showed the causality of 25 immune cell subtypes on SA was essential.
Abbreviations: SA, Spontaneous abortion; CI, confidence interval; IVW, Inverse variance weighted; SNPs, single-nucleotide polymorphisms.

CD8− T cell (RC), Granulocyte TBNK, CD28− CD127− CD25++ CD8+ T cell, Central Memory CD4+ T cell, CD25 on transitional B cell, CD25++ CD8+ T cell, CD25 on IgD+ CD38+ B cell and IgD+ CD24+ B cell were positively causally associated with the risk of SA (Figures 4 and 5), showing no evidence of heterogeneity, horizontal pleiotropy, or reverse causality (Supplementary Tables S4 and S5).

Causal Effects Between Inflammatory Cytokines and Immune Cells

To determine the causal relationship between the two inflammatory cytokines and 25 immune cell traits that have crucial causal effects on SA, we conducted further MR analysis. The results revealed that elevated PDL1 levels were positively associated with CD45 on GR-MDSC (OR = 1.422, 95% CI [1.060,1.908], p = 0.019) (Supplementary Table S6). This finding was validated using BWMR analysis, which confirmed the positive association of PDL1 with the level of CD45 on GR-MDSC (OR = 1.458, 95% CI [1.076,1.977], p = 0.015) (Supplementary Table S7).

Moreover, both MR-IVM and BWMR analyses confirmed a causal link between TNF-β and Terminally Differentiated CD4− CD8− T cell (MR-IVM: OR = 0.928, 95% CI [0.868,0.992], p = 0.027; BWMR: OR = 0.926, 95% CI [0.864,0.993], p = 0.031). Additionally, TNF-β levels was negatively correlated with CCR2 on CD14+ CD16+ monocytes (MR-IVW: OR= 0.935, 95% CI [0.882, 0.990], p = 0.022; BWMR: OR= 0.933, 95% CI [0.877, 0.993], p = 0.028) (Supplementary Tables S6 and S7). Sensitivity analyses confirmed the robustness of these associations, with no evidence of heterogeneity, horizontal pleiotropy, or reverse causality (Supplementary Tables S6 and S8).

Mediation Mendelian Randomization Analysis

Combining our previous findings on the causal links among inflammatory cytokines and SA and immune cells, we infer that GR-MDSC, Terminally Differentiated CD4− CD8− T cells, and CCR2 on CD14+ CD16+ monocytes likely function as mediators in the pathway from inflammatory cytokine to SA. As presented in Supplementary Table S9, mediation MR analysis demonstrated that GR-MDSC could be a mediator for the relationship between PDL1 and SA, with a mediation proportion of 14.05%. Additionally, Terminally Differentiated CD4− CD8− T cells also played a mediation role,

Exposure	Exposure_id	Outcome	Method	P.value	OR (95% CI)
CD38 on CD20- B cell	GCST90001809	SA	BWMR	0.011	0.939 (0.895 - 0.986)
CD25 on IgD- CD38dim B cell	GCST90001789	SA	BWMR	0.001	0.943 (0.910 - 0.977)
CD127 on CD8+ T cell	GCST90001927	SA	BWMR	0.010	0.953 (0.919 - 0.989)
CD3 on Natural Killer T cell	GCST90001848	SA	BWMR	0.024	0.962 (0.929 - 0.995)
CD45 on GR- MDSC	GCST90002047	SA	BWMR	0.021	0.963 (0.932 - 0.994)
CD80 on granulocyte	GCST90002040	SA	BWMR	0.005	0.965 (0.941 - 0.989)
CD4+ T cell	GCST90001590	SA	BWMR	0.038	0.968 (0.939 - 0.998)
CCR2 on CD14+ CD16+ monocyte	GCST90001992	SA	BWMR	0.001	0.971 (0.954 - 0.988)
CD28+ CD45RA+ CD8+ T cell(AC)	GCST90001690	SA	BWMR	0.034	0.994 (0.989 - 0.999)
CD28+ CD45RA+ CD8+ Tcell(RC)	GCST90001688	SA	BWMR	0.013	0.996 (0.993 - 0.999)
IgD+ CD38dim B cell	GCST90001394	SA	BWMR	0.042	1.020 (1.001 - 1.039)
CD28- CD8dim T cell	GCST90001662	SA	BWMR	0.034	1.019 (1.001 - 1.036)
CD8 on HLA DR+ CD8+ T cell	GCST90002060	SA	BWMR	0.040	1.023 (1.001 - 1.045)
CD24 on IgD+ CD38- B cell	GCST90001766	SA	BWMR	0.025	1.029 (1.004 - 1.056)
CD28 on activated CD4 regulatory T cell	GCST90001902	SA	BWMR	0.038	1.031 (1.002 - 1.061)
CD3 on Effector Memory CD4+ T cell	GCST90001843	SA	BWMR	0.035	1.028 (1.002 - 1.056)
Terminally Differentiated CD4- CD8- T cell	GCST90001573	SA	BWMR	0.036	1.029 (1.002 - 1.057)
CD28- CD4- CD8- T cell(RC)	GCST90001694	SA	BWMR	0.045	1.036 (1.001 - 1.073)
Granulocyte TBNK	GCST90001652	SA	BWMR	0.017	1.042 (1.007 - 1.078)
CD28- CD127- CD25++ CD8+ T cell	GCST90001673	SA	BWMR	0.025	1.054 (1.007 - 1.104)
Central Memory CD4+ T cell	GCST90001539	SA	BWMR	0.014	1.045 (1.009 - 1.083)
CD25 on transitional B cell	GCST90001795	SA	BWMR	0.004	1.055 (1.018 - 1.094)
CD25++ CD8+ T cell	GCST90001679	SA	BWMR	0.020	1.053 (1.008 - 1.101)
CD25 on IgD+ CD38+ B cell	GCST90001783	SA	BWMR	0.010	1.056 (1.013 - 1.100)
IgD+ CD24+ B cell(RC)	GCST90001439	SA	BWMR	0.039	1.059 (1.003 - 1.118)

Figure 5 Bayesian-weighted Mendelian randomization analysis validates the causal relationship between SA and the 25 immune cell subtypes.
Abbreviations: SA, Spontaneous abortion; CI, confidence interval; BWMR, Bayesian-weighted Mendelian randomization.

accounting for 5.81% of the mediation effect in the causal pathway from TNF-β to SA. A very weak mediating effect of CCR2 on CD14+ CD16+ monocyte in the pathway of TNF-β affecting SA was observed, with a mediation proportion of 0.55%.

Pathway and Functional Enrichment Analysis

To investigate the potential mechanism by which PDL1 and GR-MDSC reduce the risk of SA, we identified the genes most significantly associated with their representative SNPs using FUMA, and then performed pathway and functional enrichment analysis. BPs, CCs, and MFs terms were depicted in Figure 6A–C, showing that PDL1 and GR-MDSC representative SNP's mapping genes were significantly enriched in categories such as embryonic skeletal system development, regionalization, anterior/posterior pattern specification, lymphangiogenesis, hormone activity and nuclear localization sequence binding. Additionally, KEGG enrichment analysis demonstrated that they were significantly enriched in Signaling pathways regulating pluripotency of stem cells, Cell adhesion molecules, Galactose metabolism and Nicotinate and nicotinamide metabolism (Figure 6D).

Similarly, TNF-β and Terminally Differentiated CD4− CD8− T cells may exert protective effects on SA through the following pathways such as skin development, regulation of immune effector process, regulation of cell killing, ATPase-

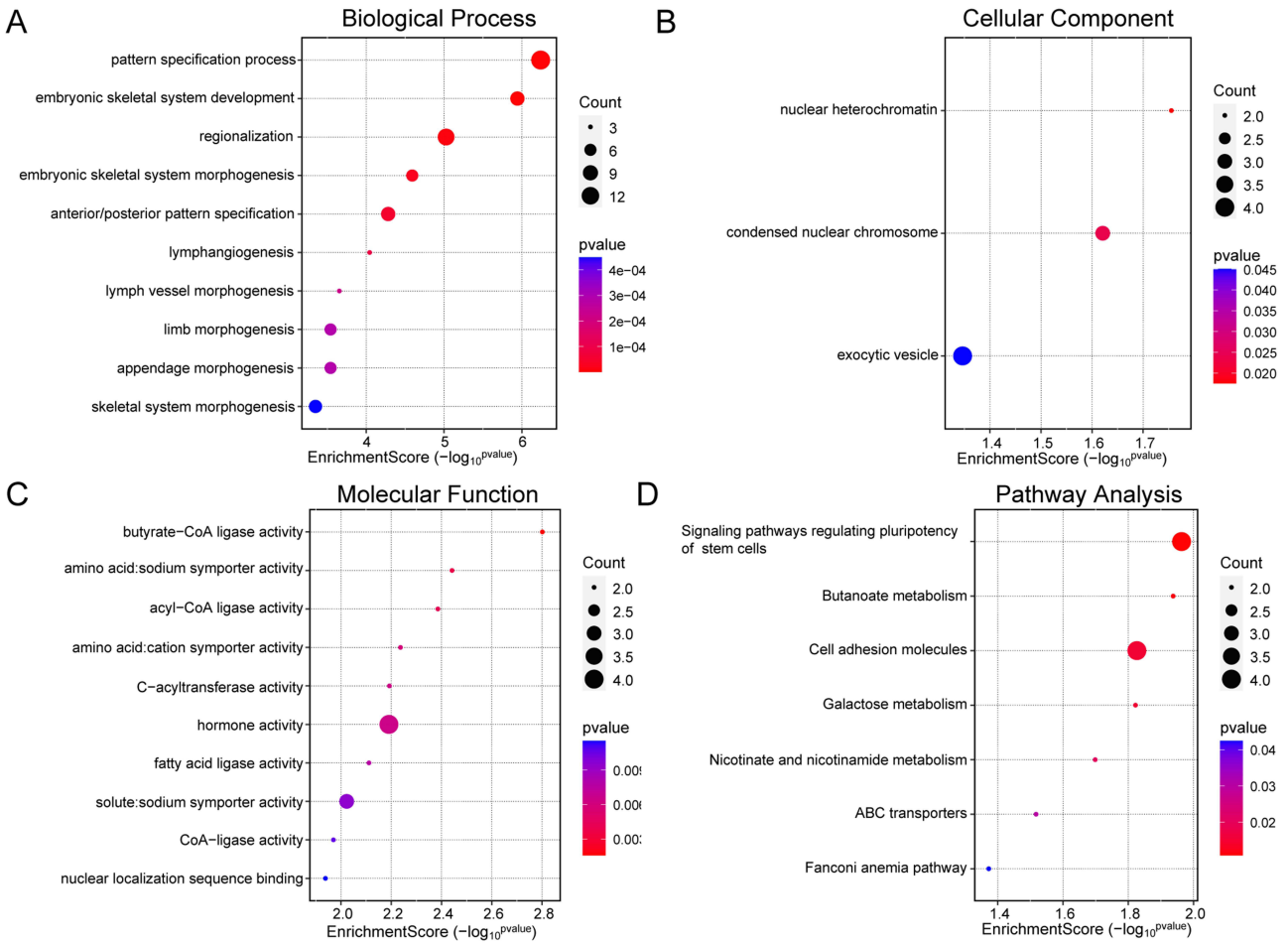

Figure 6 Gene Ontology analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the mapping genes of representative SNPs of PDL1 and GR-MDSCs. (**A**) Enriched GO terms for biological process; (**B**) Enriched GO terms for the cellular component; (**C**) Enriched GO terms for molecular function; (**D**) Enriched KEGG pathways.

Abbreviations: SNPs, single-nucleotide polymorphisms; PDL1, Programmed cell death 1 ligand 1; GR-MDSCs, Granulocytic Myeloid-Derived Suppressor Cells; KEGG, Kyoto Encyclopedia of Genes and Genomes.

coupled transmembrane transporter activity, Estrogen signaling pathway and complement and coagulation cascades (Figure 7A–D).

Discussion

Inflammatory cytokines, which are essential signaling molecules, work in concert with immune cells to play a vital role in maternal and fetal immune tolerance during normal pregnancy.[30] Recent studies have increasingly focused on the association between maternal immune abnormalities and SA, as well as recurrent miscarriages.[31–33] However, due to the complexity of immune status changes during pregnancy, real-time dynamic monitoring presents considerable challenges. Furthermore, managing the confounding variables that may affect individual immune variability is also a complex task.[34] To date, no MR study has comprehensively explored the causal connection between immune-related factors and SA. Our study aims to address these gaps. By leveraging GWAS summary data and employing genetic variations as IVs in MR analyses, we effectively minimized confounding influences, allowing us to objectively infer the etiological roles of inflammatory cytokines and immune cells in SA. Our findings identified two inflammatory cytokines (PDL1 and TNF-β) and 25 immune cell phenotypes (including Terminally Differentiated CD4− CD8− T cell and CD8 on HLA-DR+ CD8+ T cells) as crucial causal factors for SA. These cytokines and immune cell phenotypes are known to play key roles in immune regulation and inflammatory responses, which are critical in maintaining pregnancy and preventing immune-

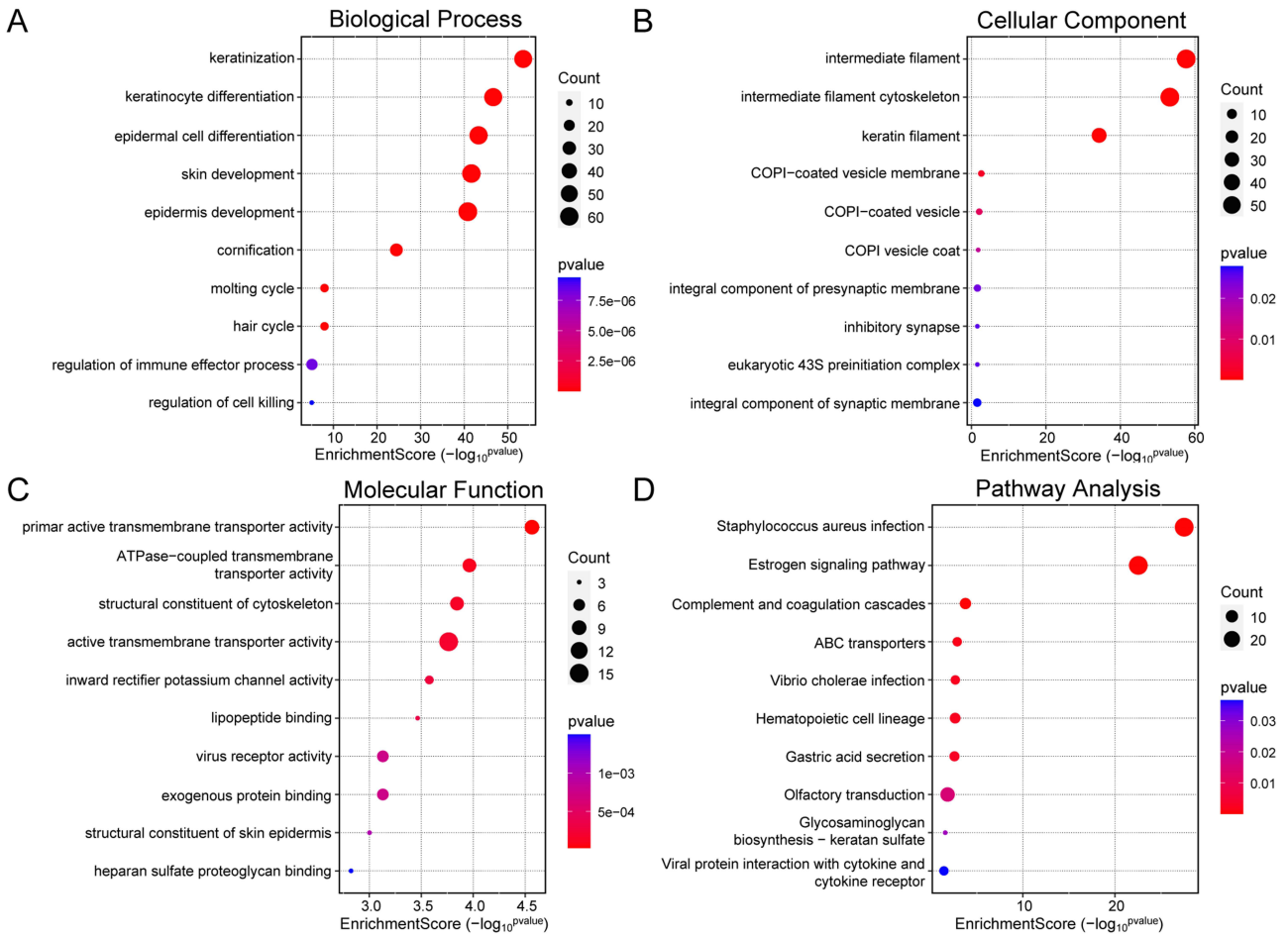

Figure 7 Gene Ontology analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the mapping genes of representative SNPs of TNF-β and Terminally Differentiated CD4- CD8- T cells. (**A**) Enriched GO terms for biological process; (**B**) Enriched GO terms for the cellular component; (**C**) Enriched GO terms for molecular function; (**D**) Enriched KEGG pathways.

Abbreviations: SNPs, single-nucleotide polymorphisms; TNF-β, Tumor necrosis factor – beta; KEGG, Kyoto Encyclopedia of Genes and Genomes.

mediated fetal rejection. Additionally, our results indicated that specific immune cell subtypes, such as GR-MDSC and Terminally Differentiated CD4− CD8− T cells, which act as potential intermediaries.

Programmed cell death-1 (PD1) and its ligand PDL1 are prominent immunosuppressive checkpoints in the tumor microenvironment by inhibiting the function of immune cells and thus promoting the immune evasion of tumors, making them focal points for cancer immunotherapy.[35] Unlike the tumor microenvironment, establishing a maternal-fetal immune tolerance microenvironment in early pregnancy is paramount for the maintenance of gestation.[36,37] PD1 and PDL1 may serve as crucial positive regulators during this period. Zhang et al demonstrated that in patients receiving embryo transfer (ET), the concentration of serum PDL1 was significantly elevated on the 23rd and 30th days after ET compared to the levels on the day of ET in the live birth cohort. In contrast, this elevation in serum PDL1 concentration was not observed in the cohort that experienced SA.[38] Another study observed a similar phenomenon, showing that patients with SA had significantly lower levels of serum PDL1 compared to subjects with normal pregnancies and legally induced abortions.[39] Zhang et al further proposed that defects in the PD1/PDL1 axis during pregnancy lead to SA by promoting M1 differentiation and inhibiting M2 differentiation in macrophages.[40] Consistent with their results, our MR analysis confirmed the causal relationship between PDL1 and SA, identifying PDL1 as a protective factor. However, no such association was found for PD1. Additionally, the depletion of MDSCs has been implicated in the dysregulation of immune responses during pregnancy, leading to SA.[41] This phenomenon has garnered increasing attention. GR-MDSCs contribute to immune tolerance and vascular remodeling of the placenta-uterine unit in patients with recurrent

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miscarriages, thereby reducing the incidence of SA.[42] Of note, both PDL1 and GR-MDSC have immunosuppressive or immune-tolerant effects during pregnancy. Our mediation MR study suggested that GR-MDSCs serves as mediators in the causal relationship between PDL1 and SA. The current evidence elucidating the mechanisms by which PDL1 influences pregnancy outcomes through or in conjunction with GR-MDSCs remains insufficient. Therefore, further research is warranted to explore this aspect in greater depth.

We preliminarily explored the potential mechanisms through which PDL1 and GR-MDSCs cooperate to influence the incidence of SA by analyzing the mapping genes of representative SNPs of PDL1 and GR-MDSCs using GO and KEGG tools. The analysis revealed that these genes predominantly participate in signaling pathways such as regulating stem cell pluripotency, cell adhesion molecules, hormone activity and galactose metabolism and ABC transporters, all of which are crucial for successful pregnancy.[43–45] For example, Gershoni et al identified a significant association between the ABC transporter family, particularly the ABCA9 gene, and early abortion rates through a GWAS analysis.[46] The study suggested that ABC transporters regulate the exchange of substances within the placenta, preventing the accumulation of harmful substances, thereby protecting the embryo from the risk of early abortion.

Normal pregnancy favors the induction of the Th2 type of immune response, whereas the Th1 type immune response often results in adverse pregnancy outcomes.[47] Both TNF-α and TNF-β are inflammatory cytokines of the TNF family. The role of TNF-α in SA has been extensively studied, while the effect of TNF-β on SA has been less explored. Makhseed et al reported that peripheral blood mononuclear cells from women with recurrent miscarriage secrete higher concentrations of Th1 cytokines, including IL-2, IFN-γ, TNF-α, and TNF-β.[48] However, TNF-β was not detected in the serum samples from patients with unexplained recurrent spontaneous abortion (URSA) or those with normal deliveries in their subsequent small-scale study.[49] Our MR results suggested that the elevated level of TNF-β may reduce the risk of SA, which appears to conflict with previous findings. The reasons for this discrepancy may include: advancements in current technology have made detecting inflammatory proteins easier, and our study included a larger cohort of participants compared to previous research. Consequently, our results that TNF-β act as a protective factor against SA is more plausible and suggestive. CD4− CD8− T cells, also known as double-negative regulatory T cells, constitute a rare subset of peripheral T cells. Despite their scarcity, they play a critical role in modulating immune tolerance within the female reproductive system. It is hypothesized that dysregulation of CD4− CD8− T cell may contribute to ovulatory dysfunction, implantation failure, and pregnancy loss.[50] Our results showed that the increased levels of CD4− CD8− T cell were a risk factor for SA. Currently, research on the relationship between CD4− CD8− T cells and SA is limited. The available evidence does not provide a definitive conclusion regarding the role of CD4− CD8− T cell in recurrent spontaneous abortion (RSA).[51,52] The investigation into the relationship between CD4− CD8− T cell and SA remains in the early stages and requires extensive further research.

The functional enrichment analysis demonstrated that TNF-β and terminally differentiated CD4− CD8− T cells may be involved in the occurrence of SA through pathways such as ABC transporters, regulation of cell killing, estrogen signaling pathway and complement and coagulation cascades. Consistent with previous studies, it has been shown that the estrogen signaling pathway, mediated by estrogen receptor α (Erα) in the oviduct, regulates the balance of proteases and protease inhibitors, which is essential for sperm migration and embryo development.[53] Disruption of this pathway leads to impaired sperm migration and embryo death. Regarding the complement and coagulation cascades pathway, it has been observed that alterations in this pathway may significantly affect pregnancy outcomes.[54,55] Compared to the endometrium of fertile controls, the complement and coagulation cascades pathway was differentially expressed in the endometrium of women with unexplained infertility and recurrent pregnancy loss.[56] Activation of this pathway may cause immune dysregulation and impaired endometrial perfusion, potentially impairing embryo implantation and leading to miscarriage. These findings highlight the potential contribution of TNF-β and terminally differentiated CD4− CD8− T cells through these pathways in the pathogenesis of SA, emphasizing the importance of further studies to elucidate the mechanisms involved.

In this study, we found that elevated levels of CD3 on NKT cells may reduce the risk of developing SA, while increased levels of CD8 on HLA-DR+ CD8+ T cells and CD3 on Effector Memory CD4+ T cells could be risk factors for SA, consistent with previous research. Rezayat et al highlighted the crucial role of NKT cells in maternal immune tolerance during early pregnancy. They observed that the administration of low-dose prednisolone significantly increased

CD3+CD8+CD56+ NKT cells in the peripheral blood of women with RSA, which is beneficial for pregnancy.[57] Additionally, previous research has identified HLA-DR+ CD8+ T cells as key biological markers in autoimmune diseases, such as systemic lupus erythematosus, and elevated levels have been observed in women with obstetric antiphospholipid syndrome.[58] A cross-sectional case-control study demonstrated that women with URSA had significantly higher percentages and absolute counts of HLA-DR+ CD8+ T cells compared to healthy controls.[59] A higher percentage of HLA-DR+ CD8+ T cells indicates an inflammatory condition combined with immune dysregulation, which may suggest an increased risk of abortion. Muyayalo et al used flow cytometry to assess the percentages of peripheral blood immune cells in nulligravida women, women with a history of normal pregnancy, and women with a history of pregnancy loss (PL). The study found that women in the PL group exhibited significantly different immune cell subset proportions compared to the other two groups, including central memory CD4+ T cells, terminally differentiated CD4+ T cells, mature NK cells, Vδ1+ T cells, effector memory CD4+ T cells (CD3+ CD4+ CD45RA- CCR7-), and Vδ2+ T cells.[60] These changes in immune cell subset proportions may disrupt maternal-fetal immune tolerance, potentially increasing the risk of abortion.[61] While direct evidence linking these immune cell subsets to SA is limited, these studies indirectly support their involvement in pregnancy outcomes.

It must be acknowledged that this study has certain limitations. First, the relationship between SNP loci and functional trait is not yet fully elucidated, making it challenging to completely detect genetic pleiotropy. Second, since the study is based on a European population database, its applicability to other ethnic groups remains uncertain, and caution is warranted when extrapolating these conclusions to different populations. Third, the GWAS population samples used were not large enough, and no genetic loci for inflammatory proteins or immune cells met the significance threshold of P-value < 5e−8. Fourth, the GWAS database for the inflammatory cytokines and immune cells were included both males and female, which may affect the accuracy of the results. Currently, no female-specific GWAS dataset is available. Although vast GWAS datasets are now available, further advancement in MR research will require even larger sample sizes, more diverse populations, and tailored to specific research questions.

Conclusion

In conclusion, this bidirectional MR study comprehensively assessed the causal relationship between inflammatory cytokines, immune cell subtypes and SA. Elevated levels of PDL1 and TNF-β may reduce the risk for SA. Moreover, multiple immune cells phenotypes, including Terminally Differentiated CD4− CD8− T cell, CD8 on HLA-DR+ CD8+ T cells, CD3 on NKT cells and CD3 on Effector Memory CD4+ T cells, demonstrated significant causal associations with SA occurrence. These findings offer novel insights into the immunological etiology of SA from a genetic perspective and suggest potential clinical implications for pregnancy preparation.

Data Sharing Statement

The inflammatory cytokines GWAS summary data underlying this article are available in the GWAS Catalog (https://www.ebi.ac.uk/gwas/), and can be accessed with accession numbers GCST90274758 to GCST90274848; The immune cell GWAS summary data underlying this article are available in the GWAS Catalog (https://www.ebi.ac.uk/gwas/) under the study accession numbers GCST90001391 to GCST90002121; The GWAS summary data for spontaneous abortion is derived from the FinnGen (https://www.finngen.fi/fi.finngen_R10_O15_ABORT_SPONTAN).

Ethical Approval

All participating studies involved in the GWAS obtained informed consent from the study populations. As we utilized publicly available datasets to conduct MR, no additional ethics approval was required.

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Disclosure

The authors report no conflicts of interest in this work.

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