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Original Article

Immune cells mediated the causal relationship between perturbational phenotyping of human blood cells and neuropathy pain: A two-sample and mediated mendelian randomized study

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Abstract

Current research reveals a complex relationship between blood cells(BC) and neuropathic pain(NP), though the underlying biological mechanisms remain unclear. This study applies Mendelian randomization (MR) to investigate causal relationships between BC and three major types of NP: diabetic peripheral neuropathy(PDPN), postherpetic neuralgia(PHN), and trigeminal neuralgia(TN). We also explore the potential mediating role of immune cells in these associations. We employed a two-sample, two-step Mendelian randomization study using the inverse variance weighted method to investigate the causal effect of BC on three major types of NP, as well as the mediating role of immune cells in the association between BC and NP. Additionally, we utilized a two-step Mendelian randomization design to explore the mediating effect of immune cells. We identified 13 distinct blood cell phenotypes under various perturbation conditions that have a significant causal relationship with NP. Additionally, we discovered 127 immune cells that exhibit a notable causal connection with NP. Through Mendelian Randomization (MR) and two-step Mendelian Randomization analyses, we found the following results: Three blood cell phenotypes were associated with PDPN, three with PHN, and seven with TN, with platelet, red blood cell, monocyte, and neutrophil responses showing significant correlations with NP risks. Immune cell analyses revealed 36 phenotypes increasing and 31 decreasing PDPN risk, 16 increasing and 21 decreasing PHN risk, and 18 increasing and 13 decreasing TN risk, with HLA DR on DCs, PB/PC AC, and CD39+ CD4+ %T cell showing the strongest associations, respectively. Mediation analysis identified immune cells, such as CD39+ resting Treg and HLA DR+ CD4+ %lymphocyte, mediating PBC effects on NP risks. Sensitivity analyses confirmed no significant heterogeneity or pleiotropy, and reverse MR analyses found no reverse causal relationships. This study provides new evidence for the causal relationship between blood cell phenotypes and neuropathic pain and proposes immune factors with potential mediating effects. However, this finding needs to be further demonstrated by more extensive clinical studies.

Keywords: Blood cell phenotypes, Immune cells, Neuropathic pain, Mendelian randomization study.

1. Introduction

Neuropathic pain arises from damage or disease affecting the somatosensory nervous system[1], either peripherally induced neuropathic pain (pNP) or neuropathic pain (NP). Peripheral neuropathic pain is more common, typically resulting from peripheral nerve injury. Examples[2-4] of neuropathic pain include painful diabetic peripheral neuropathy (PDPN), trigeminal neuralgia (TN), and postherpetic neuralgia (PHN). Neuropathic pain is a form of chronic pain that significantly impacts patients' quality of life. Drug-induced analgesia is currently the primary method for alleviating neuropathic pain[1, 5], but despite advances in pain research, treating neuropathic pain remains challenging. There is no consensus on the most appropriate therapeutic regimen.

Previous genome-wide association studies (GWAS) primarily focused on complete blood count (CBC), describing clinical parameters of white blood cells, red blood cells, and platelets, as well as the genetic architecture of hematopoiesis and blood diseases[6-8]. These studies did not account for the dynamic responses of blood cells to environmental conditions, which are likely to influence disease development, progression, and prevention. Treating whole blood with various stressors or stimuli in vitro can identify differential cellular responses and uncover novel disease-related endophenotypes. Expanding this phenotypic space could reveal traits determined by common alleles with large effect sizes, thus improving the efficiency of target identification and enhancing the prediction of disease occurrence[9]. Currently, no studies have investigated the association between blood cells and disease under altered environmental conditions. Most studies have observed

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whether static changes in blood cells influence disease and whether these changes affect immune cell phenotypes and thus contribute to disease onset and progression.

Recent studies have explored the mediating role of immune cells in the relationship between gut microbiota and NP, emphasizing the crucial role of immune cells in NP[10]. However, the association between perturbation blood cell phenotypes (PBC) under changing environmental conditions and diseases, particularly the key role of immune cells in this context, has not been investigated. Previous research indicates that decreased expression of IBA1+ macrophages is associated with the development and progression of type 1 diabetes neuropathy[11, 12]. Activating A3AR on CD4+ T cells to release IL-10 has been shown to reduce the excitability of dorsal root ganglion (DRG) neurons[13]. The frequency of VZV-specific PD-1+ CD4+ T cells and the amount of TNF-α produced by VZV-specific T cells are higher in patients without PHN[14]. The proportion of helper T cells (CD3+, CD4+) after neuropathic pain treatment is greater than before treatment, potentially alleviating disease progression[15]. Changes in neutrophil activation are linked to the occurrence of PHN. There is a negative correlation between the frequency of NK cells and mechanical pain sensitivity (MPS), suggesting that a high frequency of NK cells may prevent central sensitization and play a protective role in the neuroinflammatory cascade[16]. The depletion of regulatory T cells (Tregs) delays spontaneous pain recovery, indicating their potential key role in neuropathic pain[17]. While these studies highlight critical factors related to immune cells in the development of neuropathic pain, there is a lack of research on whether changes in blood cell phenotypes (including red blood cells and platelets) in the microenvironment are interconnected with disease and whether these changes influence the phenotype of immune cells, subsequently affecting the occurrence and progression of the disease.

Mendelian randomization (MR) uses genetic variation as an instrumental variable (IV) to infer causal relationships between exposures and outcomes. This method leverages genetic variations, such as single nucleotide polymorphisms (SNPs), as IVs to represent exposure factors. Since these SNPs are randomly distributed and independent of environmental factors and other confounders[18, 19], MR is a method that controls for potential confounding and avoids reverse causality bias. MR provides rigorous interpretations of causal relationships between complex factors. In this study, we first performed univariate bidirectional MR analyses to assess the effects of blood cell phenotypic traits on neuropathic pain susceptibility under various perturbation conditions. We then applied two-step MR analyses to screen for immune phenotypes that may serve as potential mediators of the relationship between changes in blood cell phenotypes in the microenvironment and neuropathic pain.

2. Materials and methods

2.1. Study design

We employed the mediation MR method to investigate the causal relationships between peripheral blood cell phenotypes, immune cells, and three major NP conditions: postherpetic neuralgia (PHN), painful diabetic peripheral neuropathy (PDPN), and trigeminal neuralgia (TN) (Figure 1). This analysis aims to establish the causal effect of peripheral blood cells on immune cells, and subsequently, their impact on these neuropathological conditions. We employed further analysis to elucidate significant causal links.

2.2. GWAS summary data sources

The summary statistics of blood cells were retrieved from the largest genome-wide association studies (GWAS) of 2,600 individuals exploring all blood perturbation profiles, identifying more than 100 genomic loci significantly associated with at least two blood perturbation readouts. The blood cells were adapted to a widely-used whole-blood cytometry analyzer (Sysmex XN-1000) to systematically profile peripheral blood from over 4,700 study participants (donors) under 37 conditions (36 perturbations and baseline), genotyped more than 2,600 donors and performed GWAS for all blood perturbation profiles[9]. Summary statistics of blood cells were acquired on the GWAS Catalog (https://www.ebi.ac.uk/gwas/) under the study accession numbers GCST90257015 to GCST90257105.

Summary statistics for all immunologic characteristics in the GWAS catalog (accession numbers GCST90001391 to GCST90002121) are readily accessible[20].

The GWAS included 3,757 non-overlapping individuals of European descent and utilized a high-density array based on a reference panel of Sardinian sequences[21], estimating approximately 22 million SNPs. Correlations were tested while controlling for covariates such as age, age², and sex. A total of 731 immunophenotypes were assessed, encompassing relative cell counts (RC), morphological parameters (MP), absolute cell counts (AC), and median fluorescence intensities (MFI), which indicate surface antigen levels. Among these, MP features included CDC and TBNK panels, while MFI, RC, and AC features covered B cells, CDC, T cell maturation stages, myeloid cells, monocytes, and TBNK (which includes T cells, B cells, and natural killer cells).

Neuropathic pain is mostly caused by peripheral nerve damage, and peripheral neuropathic pain is often related to PDPN. We selected PDPN as a representative. Below are

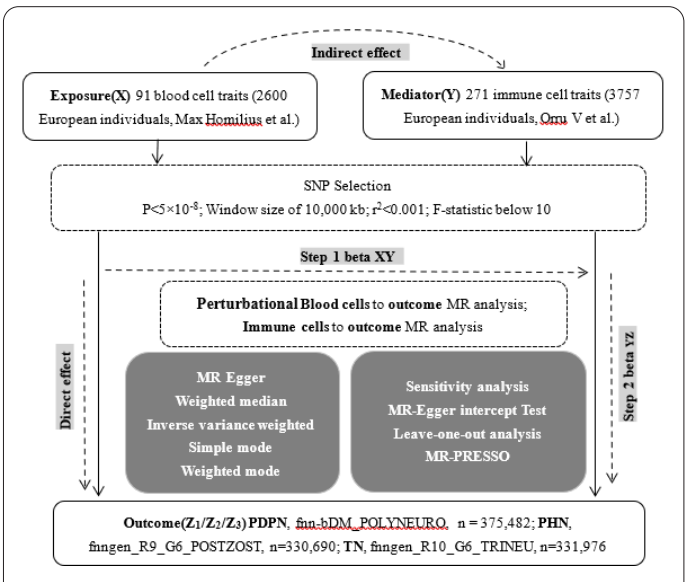

Fig. 1. This study designs a flowchart. A two-step Mendelian randomization study of PBC on NP mediated by immune cells. Mediation analysis of the effect of PBC. *PBC* Perturbational blood cells, *NP* neuropathic pain, *MR* Mendelian randomization, *SNP* single nucleotide polymorphism.

the sources of the three types of data[1]. Data (Table 1) on (PDPN[4], fnn-bDM_POLYNEURO, n = 375,482), postherpetic neuralgia (PHN[3], fnngen_R9_G6_POSTZOST, n =330,690), and trigeminal neuralgia (TN[2], fnngen_R10_G6_TRINEU,331,976) were obtained from the FinnGen consortium's GWAS summary data, which is publicly available on the FinnGen website[22-24]. Since this study relies entirely on publicly accessible aggregated data, no additional ethical approval or participant consent was necessary.

2.3. Instrumental variable selection and data harmonization

To ensure high-quality results, we implemented rigorous quality control procedures to select SNPs that were significantly associated with the target phenotypes(P-value < 1e-5) as instrumental variables (IVs). Specifically, we utilized measurements of blood cell reactivity under various chemical and physical perturbation conditions[9], correlating SNPs with these blood cell phenotypes' GWAS results to ensure the selection of SNPs related to the exposure variables (e.g., blood cell responses under different environments). The selected SNPs were clustered based on linkage disequilibrium (LD) to ensure that each instrumental variable was independent. Clustering was performed using a 10,000 kb window and an $r^2 < 0.001$ threshold, with LD data sourced from European samples in the 1000 Genomes Project[25]. This step ensured that the chosen SNPs were independently distributed across different chromosomal regions, avoiding issues of multicollinearity due to LD. If certain exposure SNPs could not be found in the dataset, we selected highly correlated proxy SNPs to include as much data as possible in the analysis. All palindromic SNPs and those with ambiguous sequences were excluded to prevent introducing unclear directionality of effects in the Mendelian randomization analysis. To ensure the robustness of the instrumental variables, we evaluated the strength of each SNP using the F-statistic, calculated as the ratio of the squared effect size (β) to the standard error. SNPs with an F-statistic less than 10 were considered weak instruments and were excluded from subsequent analyses. To further assess the strength of the instrumental variables and reduce weak instrument bias, we validated each SNP using the F-statistic. SNPs with an F-statistic greater than 10 were considered strong instruments and retained for the final MR analysis.

2.4. Univariate MR analysis

To predict the effects of various PBCs on PN, we employed three complementary methods for univariable MR analysis: IVW, MR-Egger, and the weighted median method. The IVW method is considered the primary causal estimation approach and can provide accurate results when all selected SNPs are valid IVs[26]. The MR-Egger method produces consistent causal estimates under the InSIDE assumption, independent of the instrument strength of direct effects, even if the genetic IVs are invalid. However, it should be noted that this method is imprecise and susceptible to influences from peripheral genetic variation[27]. On the other hand, the weighted median method calculates the weighted median of Wald ratio estimates without relying on the InSIDE assumption and is robust to horizontal pleiotropy bias. Compared to the MR-Egger method, the weighted median method exhibits lower Type I error and higher causal estimation power[28]. A causal relationship between the exposure and the outcome is considered when the IVW analysis results in $P<0.05$, and there is consistency across the three methods. All statistical analyses and data visualizations were performed using the R programming software (version 4.4.1), including the "TwoSampleMR" R package (0.5.7) and the "MRPRESSO" R package (PMID: 24114802). The "forestplot" R package (version 1.1.1) was used to generate a forest plot.

2.5. Mediated MR analysis

Our analysis focused on the impact of PBC on PN development. We employed a two-step MR method to investigate potential immune phenotypes that could serve as mediators between PBC and PN patients. The two-step mediation MR technique can be compared to the product of coefficients method. It involves calculating two MR estimates: the causal effect of the exposure on the mediator, and the causal effect of the mediator on the outcome. By multiplying these two estimates, we obtain an estimate of the indirect effect. This advanced analytical approach allows us to decompose the total effect into indirect (mediated by immune cells) and direct (independent of the mediator) components[29], as illustrated in Figure 1. Specifically, the total impact of BC on NP is divided into (1) the direct effect of BC on NP, and (2) the indirect effect mediated by immune cells. This method enables us to estimate the percentage of the total effect accounted for by the mediation effect by comparing the size of the indirect effect to the total effect.

2.6. Statistical analysis

All statistical analyses were performed using R version 4.4.1 (The R Foundation for Statistical Computing, Vienna, Austria). Two-sample Mendelian randomization (MR) analyses were conducted using three specialized R packages: "TwoSampleMR" for MR analysis, "VariantAnnotation" for genetic variant annotation, and "ieugwasr" for accessing GWAS summary statistics. Five distinct MR methods were employed to assess cau-

Table 1. Details of the studies included in the Mendelian randomization analyses.

Data From	Phenotype	Sample size	Case	Control	Ancestry
GWASID(GCST90257015-GCST90257105)	Blood Cell traits	-	-	-	European
GWASID(ebi-a-GCST90001391–ebi-a-GCST90002121)	Immune cell traits	-	-	-	European
fnn-R9-DM_POLYNEURO	PDPN(painful diabetic polyneuropathy)	375482	1048	374434	European
fnngen_R9_G6_POSTZOST	PHN(post-herpetic neuralgia)	330690	313	330377	European
fnngen_R9_G6_TRINEU	TN(trigeminal neural gia)	331976	1599	330377	European

sal relationships: MR Egger[27], weighted median[28], inverse variance weighted (IVW)[26], simple mode[30], and weighted mode[28]. Among these, IVW served as the primary analytical approach due to its superior precision and robustness in causal estimation. Statistical significance was determined at $P < 0.05$, indicating a significant association between exposure and outcome variables. To evaluate heterogeneity across genetic variants, we implemented Cochran's Q statistic based on both IVW and MR Egger methods. Potential pleiotropic effects were assessed through two complementary approaches: the MR-Egger intercept test and the MR pleiotropy residual sum and outlier (MR-PRESSO) method, the latter being executed using the "MR-PRESSO" package. The MR-PRESSO framework was additionally utilized to correct for horizontal pleiotropy through outlier removal and to evaluate the significance of differences before and after such corrections. Sensitivity analyses were conducted to ensure the robustness of our findings. Leave-one-out analysis was systematically performed to examine the potential influence of outlying genetic variants on the overall results. Furthermore, we employed the "product of coefficients" method to quantify the indirect effect of PBC on NP risk through potential mediators, with standard errors for these indirect effects calculated using the delta method.

3. Result

3.1. Causal effects between neuropathic pain and blood cell phenotypes

We used the IVW method as the main analysis in two-sample MR analysis and performed Mendelian randomization on 91 types of PBC with PDPN, PHN, and TN using a threshold of P<1e-5. We identified three blood cell phenotypes associated with PDPN, three blood cell phenotypes associated with PHN, and seven blood cell phenotypes associated with TN (detailed data can be found in Supplementary Table S1).

In our study, we identified three blood cell response pathways related to the risk of PDPN. Under the conditions of using reticulocyte dye, we measured the forward scatter standard deviation (FSC SD) of red blood cells under dimethyl sulfoxide (DMSO) perturbation. We found that this response was positively correlated with the risk of developing PDPN (β=0.036, OR_IVW: 1.036, 95% CI: 1.001 to 1.073, P_IVW=0.046, P_pleiotropy=0.328). Under the conditions of using WNR dye, we measured the side fluorescence coefficient of variation (CV) of platelets after 8 hours of chloroform perturbation, and this response showed the most significant positive correlation with the risk of developing PDPN (β=0.032, OR_IVW: 1.032, 95% CI: 1.005 to 1.060, P_IVW=0.019, P_pleiotropy=0.740) (Figure 2). Additionally, under the conditions of using WDF dye, we measured the FSC SD of eosinophils under potassium chloride (KCl) perturbation, and this response was significantly negatively correlated with the risk of developing PDPN (β=-0.075, OR_IVW: 0.928, 95% CI: 0.866 to 0.994, P_IVW=0.032, P_pleiotropy=0.807).

In the MR analysis of blood cell response pathways related to PHN, we identified three blood cell phenotypes that were significantly associated with an increased risk of PHN. Under the conditions of using reticulocyte dye, we measured the side fluorescence CV of reticulocytes in the baseline state (β=0.438, OR_IVW: 1.549, 95% CI: 1.171 to 2.050, P_IVW=0.002, P_pleiotropy=0.479)(Figure 2); under the conditions of using reticulocyte dye, we measured the side scatter standard deviation (SSC SD) of platelets under Pam3CSK4 perturbation (β=0.106, OR_IVW: 1.036, 95% CI: 1.017 to 1.214, P_IVW=0.019, P_pleiotropy=0.449); under the conditions of using WDF dye, we measured the side fluorescence CV of monocytes under captopril perturbation(β=0.165, OR_IVW: 1.040, 95% CI: 1.179 to 1.337, P_IVW=0.010, P_pleiotropy=0.525), and these responses were found to be related to an increased risk of PHN. In the causal relationship with TN, we identified seven blood cell phenotypes significantly associated with TN, of which the most significantly increased risk of TN was measured under the conditions of using WDF dye, measuring the forward scatter median of neutrophils under Pam3CSK4 perturbation (β=0.159, OR_IVW: 1.173, 95% CI: 1.038 to 1.325, P_IVW=0.011, P_pleiotropy=0.699). The most significantly reduced risk of TN was measured under the conditions of using WDF dye, measuring the forward scatter CV of neutrophils under Pam3CSK4 perturbation (β=-0.103, OR_IVW: 0.902, 95% CI: 0.819 to 0.992, P_IVW=0.034, P_pleiotropy=0.938)(Figure 2). The additional five blood cell phenotypes can be found in Supplementary Table S1.

3.2. Causal effects of immune cells on PDPN

In our MR analysis of immune cells associated with PDPN, we identified 36 immune cells significantly associated with an increased risk of PDPN and 31 immune cells significantly associated with a decreased risk of PDPN (detailed information can be found in Supplementary Table S2). The strongest significant positive association was with HLA DR on DCs (from the cDC panel) (β=0.323, OR_IVW: 1.382, 95% CI: 1.253 to 1.524, P_IVW<0.001, P_pleiotropy=0.085). The strongest significant negative association was with CD25 on activated & secreting Treg (from the Treg panel) (β=-0.147, OR_IVW: 0.864, 95% CI: 0.795 to 0.938, P_IVW<0.001, P_pleiotropy=0.573) (Figure 3).

3.3. Causal effects of immune cells on PHN

In our MR analysis of immune cells associated with PHN, we identified 16 immune cells significantly associated with an increased risk of PHN and 21 immune cells significantly associated with a decreased risk of PHN(detailed information can be found in Supplementary Table S2). Among these, PB/PC AC (B cell panel)

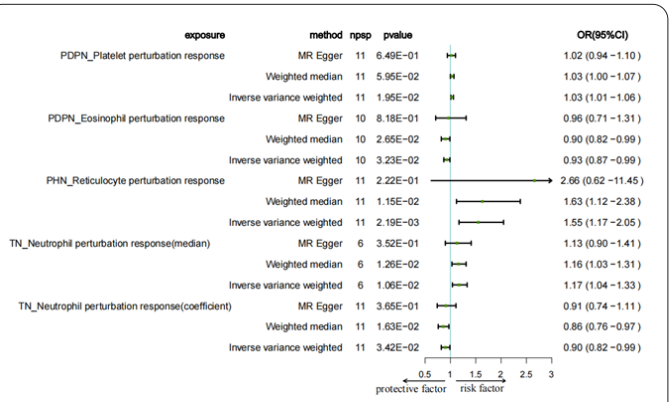

Fig. 2. Forest plot of Mendelian randomization analyses of Perturbational blood cells-associated phenotypes and NP. OR, odds ratio. 95%CI, 95% confidence interval. nsnp, number of single nucleotide polymorphisms.

showed the strongest significant positive association (β=0.148, OR_IVW: 1.160, 95% CI: 1.034 to 1.301, P_IVW=0.012, P_pleiotropy=0.398). Additionally, the most significantly reduced risk of PHN was observed with CD4 on HLADR+CD4+ (TBNK panel) (β=-0.372, OR_IVW: 0.689, 95% CI: 0.539 to 0.881, P_IVW=0.003, P_pleiotropy=0.050) (Figure 3).

3.4 Causal effects of immune cells on TN

In our MR analysis of immune cells associated with TN, we identified 18 immune cells significantly associated with an increased risk of TN(detailed information can be found in Supplementary Table S2). The strongest significant association was observed with CD39+ CD4+ %T cell (Treg panel) (β=0.075, OR_IVW: 1.078, 95% CI: 1.031 to 1.128, P_IVW=0.001, P_pleiotropy=0.312). Additionally, we identified 13 immune cells associated with a reduced risk of TN, with the most significant association being with HLA DR+ CD8br %T cell (TBNK panel) (β=-0.075, OR_IVW: 0.928, 95% CI: 0.878 to 0.979, P_IVW=0.007, P_pleiotropy=0.497) (Figure 3).

3.5. Reverse MR and mediation analysis

In the reverse MR analysis, we have not yet found evidence to confirm the existence of a reverse causal relationship between NP, immune cells, and 91 types of PBC. Notably, we conducted reverse MR analyses for PDPN, PHN, and TN, and none yielded significant results.

To identify potential intermediate immune cell phenotypes, we explored the impact of NP status: significant correlations between PBC and NP, and between immune cells and NP, to determine the associated factors for NP onset risk. We conducted a two-sample MR on blood cells and immune cells. We found that in the causal analysis of PDPN, two blood cell phenotypes were significantly associated with PDPN onset risk through the mediation of nine immune cells (detailed data in supplementary Table S3). The most significantly positive correlation was between the perturbation response of platelets exposed to chloroform for up to 8 hours (its coefficient of variation) and the proportion of CD39-expressing resting Treg cells (β=0.026, OR_IVW: 1.026, 95% CI: 1.006 to 1.047, P_IVW=0.011, P_pleiotropy=0.563). The most significantly negative correlation was between the perturbation response of red blood cells exposed to DMSO (its forward scatter standard deviation) and the proportion of double-positive T cells expressing both CD4 and CD8 (β=-0.031, OR_IVW: 0.969, 95% CI: 0.945 to 1.047, P_IVW=0.019, P_pleiotropy=0.959) (Figure 3). Additionally, we explored the

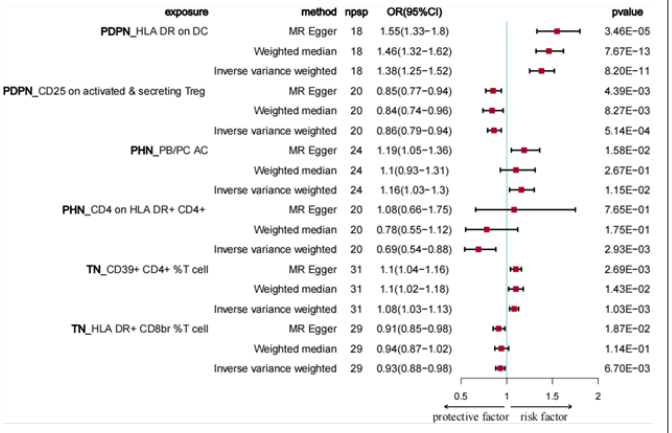

Fig. 3. Forest plot of Mendelian randomization analyses of immune cells-associated phenotypes and NP.

mediating role of immune cells in the pathway from PBC to PDPN (Table 2). Our study showed that CD39+ resting Treg %resting Treg (Treg panel) might play an opposite role in increasing PDPN risk from PBC (total effect beta: 0.032, direct effect PBC to immune cells: 0.026, direct effect immune cells to outcome: -0.099, mediation effect: -0.003); DP (CD4+CD8+) %T cell (TBNK panel) might play the same role in increasing PDPN risk from PBC (total effect beta: 0.036, direct effect BC to immune cells: -0.030, direct effect immune cells to outcome: -0.287, mediation effect: 0.027).

In the causal analysis of PHN, one blood cell phenotype was significantly associated with PHN onset risk through the mediation of one immune cell. We found that the perturbation response of monocytes exposed to Captopril (its side fluorescence coefficient of variation) was significantly correlated with the percentage of cells expressing both HLA-DR and CD4 (β=0.043, OR_IVW: 1.044, 95% CI: 1.006 to 1.083, P_IVW=0.023, P_pleiotropy=0.313) (Figure 3). We explored the mediating role of immune cells in the pathway from PBC to PHN (Table 2). Our study indicated that HLA DR+ CD4+ %lymphocyte (TBNK panel) might play an opposite role in increasing PHN risk from PBC (total effect beta: 0.165, direct effect BC to immune cells: 0.043, direct effect immune cells to outcome: -0.395, mediation effect: -0.017).

In the causal analysis of TN, four blood cell phenotypes were significantly associated with PHN onset risk through the mediation of five immune cells (detailed data in supplementary Table S2). The most significantly positive correlation was between the forward scatter median of neu-

Table 2. Mediation effect analysis. a: side fluorescence coefficient of variation of platelet in response to chloroform (8h) perturbation measured by WNR dye; **b**: forward scatter standard deviation of RBC 1 in response to DMSO perturbation measured by reticulocyte dye; **c**: side fluorescence coefficient of variation of monocyte 2 in response to captopril perturbation measured by WDF dye; **d**: forward scatter median of neutrophil 1 at baseline measured by WDF dye; **e**: forward scatter coefficient of variation of monocyte in response to Pam3CSK4 perturbation measured by WDF dye.

Exposure	Mediator	Outcome	Total effect	Direct effect	Mediation effect
Platelet perturbation response[a]	CD39+ resting Treg %resting Treg	PDPN	0.032	0.026	-0.003
Red blood cell perturbation response[b]	DP (CD4+CD8+) %T cell	PDPN	0.036	-0.030	0.027
Monocyte perturbation response[c]	HLA DR+ CD4+ %lymphocyte	PHN	0.165	0.043	-0.017
Neutrophil perturbation response[d]	CD86 on granulocyte	TN	0.155	0.373	0.044
Monocyte perturbation response[e]	DP (CD4+CD8+) AC	TN	0.128	-0.112	0.014

trophil 1 at baseline and CD86 on granulocyte (β=0.373, OR_IVW: 1.452, 95% CI: 1.172 to 1.799, P_IVW<0.001, P_pleiotropy=0.583); the most significantly negative correlation was between the forward scatter coefficient of variation of monocytes treated with Pam3CSK4 and activated DP (CD4+CD8+) T cells (β=-0.112, OR_IVW: 0.894, 95% CI: 0.830 to 0.963, P_IVW=0.003, P_pleiotropy=0.701) (Figure 3). We explored the mediating role of immune cells in the pathway from PBC to TN (Table 2). Our study indicated that CD86 on granulocyte (cDC panel) (TBNK panel) might play the same role in increasing TN risk from PBC (total effect beta: 0.155, direct effect BC to immune cells: 0.373, direct effect immune cells to outcome: 0.118, mediation effect: 0.044); DP (CD4+CD8+) AC (TBNK panel) might play the same role in increasing TN risk from PBC (total effect beta: 0.128, direct effect BC to immune cells: -0.112, direct effect immune cells to outcome: -0.127, mediation effect: 0.014).

3.6. Sensitive analysis

Conducting sensitivity analyses to evaluate heterogeneity and potential pleiotropy that could significantly violate MR analysis requirements is crucial. Horizontal pleiotropy may occur when IVs affect outcomes through pathways other than the exposure. To ensure the accuracy of our findings, we employed several methods in this study. These methods included the Cochran Q test, the MR-Egger intercept test, and the MR-Pleiotropy RESidual Sum and Outliers (MR-PRESSO) test. In the presence of heterogeneity, a Cochran Q test result with $p < 0.05$ was considered significant[31]. The MR-Egger intercept was used to assess the bias due to IVs invalidity[32]. Finally, MR-PRESSO was employed to re-examine the study for any potential horizontal pleiotropy[33]. The results showed that no horizontal pleiotropy was observed in the MR analysis of PBC and NP, as well as in the MR analysis of immune cells and NP (P_pleiotropy>0.05). Additionally, other results from MR-PRESSO and MR-Egger regression did not indicate horizontal pleiotropy, and Cochran's Q test did not show significant heterogeneity.

4. Discussion

Our study delved into the causal relationships between 91 blood cell traits, 731 immune cell traits, and NP. We used perturbation phenotypes of human blood cells under external interference to investigate how blood cell-related traits influence the development of neuropathic pain. Additionally, we explored immune cell phenotypes that could potentially act as mediators. We identified three blood cell phenotypes associated with PDPN, three blood cell phenotypes associated with PHN, and seven blood cell phenotypes associated with TN. We discovered that two blood cell phenotypes exert their effects on PDPN through nine immune cell phenotypes, originating from the B cell panel, Treg panel, TBNK panel, cDC panel, and myeloid cell panel. One blood cell phenotype influences PHN through a single immune cell phenotype from the TBNK panel. Furthermore, four blood cell phenotypes affect TN through five immune cell phenotypes from the T cell panel, TBNK panel, and cDC panel.

Perturbation blood cell phenotypes refer to the changes in blood cells' morphology and quantity under various physical, chemical, and pharmacological factors added to peripheral blood. Functional readouts based on cytometry are used to reveal underlying cellular processes, and GWAS analyses are conducted based on these induced traits[9]. This approach helps to uncover associations between induced blood phenotypes and common disease subgroups. In this study, the concepts of blood cell phenotypes and immune cell phenotypes are considered independently. Blood cell phenotypes refer to the changes in morphology and quantity of blood cells under different perturbation conditions, encompassing all types of blood cells. Immune cell phenotypes specifically refer to the types and quantities of particular immune cells, as well as the expression of certain cell surface markers. Whether the changes in these blood phenotypes are associated with immune cell phenotypes, and the role of immune cells as key factors influencing neuropathic pain, remain unexplored in existing research.

The final findings of this study mainly focus on the relationships between PBC, immune cells, and neuropathic pain. Research indicates that the most common complication in patients with sickle cell disease (SCD) is neuropathic pain, which may be mechanistically linked to changes in red blood cell morphology affecting oxidative stress, vascular occlusion, ischemia-reperfusion injury, and inflammation[34]. We discovered that, even without external stimuli or perturbations, changes in reticulocyte phenotypes increase the risk of PTN onset, potentially related to morphological changes during cell development. Existing studies have shown that platelet-rich plasma (PRP) contains natural concentrations of growth factors and immune system messengers, potentially related to tissue regeneration and repair[35], though the mechanisms by which it alleviates neuropathic pain remain unclear. It may be that factors released by platelets contribute to the reduction of neuropathic pain[36, 37]. We found that the risk of PDPN increases under chloroform-induced platelet perturbations, which may be related to the reduction in platelet count. The combined effects of neuroglial cells and cytokine-regulated inflammation play crucial roles in the development and progression of neuropathic pain. The stromal vascular fraction (SVF) of adipose tissue comprises mixed cell populations, including MSCs (mesenchymal stem cells), erythrocytes, leukocytes, and endothelial cells. By releasing cytokines, SVF exerts anti-inflammatory effects that can counteract the pathological inflammation involved in neuropathic pain and has been shown to play important roles in nerve healing and regeneration[38-40]. CD4+CD25+Foxp3+ regulatory T cells (Tregs) are endogenous immunosuppressive factors that can reduce T cell proliferation and the production of pro-inflammatory cytokines. Tregs play a role in the endogenous recovery from pain induced by neuropathies[41]. The P2X receptor plays a critical role in thrombo-inflammation mediated by platelets and neutrophils, neuroinflammation mediated by microglia, and neuropathic pain[42]. Patients with hypereosinophilic syndrome (HES) commonly exhibit peripheral neuropathy, manifesting as neuropathic pain[43, 44]. We found that, when neutrophils are perturbed by the Toll-like receptor 2 (TLR2) agonist Pam-3CSK4, their forward scatter median is associated with an increased risk of TN onset, while the forward scatter CV is associated with a decreased risk of TN onset. The relationship between changes in blood cell phenotypes and TN under different perturbation conditions may be related to changes in normal cell numbers. Under potassium chloride

perturbation, changes in eosinophil phenotypes suggest a reduced risk of PDPN, likely due to the alleviating effect of reduced eosinophil counts on peripheral neuropathy.

4.1. Mediating role of immune cells in PDPN

In the causal relationships of PDPN, we identified two blood cell phenotypes that are significantly associated with PDPN risk through the mediation of nine immune cell phenotypes. Among them, the perturbation response phenotype of platelets significantly increases the risk of PDPN through the mediation of CD39+ resting Treg cells (β=0.026, P=0.011). Treg cells play a protective role in neuroinflammation and pain regulation by inhibiting immune responses[45-47]. Therefore, changes in platelets may modulate PDPN risk by affecting Treg cell activity. Another important mediation pathway is the perturbation response phenotype of erythrocytes, which significantly reduces PDPN risk through CD4+CD8+ double-positive T cells (β=-0.031, P=0.019). CD4+CD8+ double-positive T cells have multiple functions in the immune system and may help alleviate neuropathic pain by enhancing anti-inflammatory responses[48].

4.2. Mediating role of immune cells in PHN

In the causal relationships of PHN, it was found that the perturbation response phenotype of monocytes significantly increases the risk of PHN through HLA-DR+ CD4+ lymphocytes (β=0.043, P=0.023). HLA-DR+ CD4+ cells typically play a role in antigen presentation and regulation of immune responses[10]. Their increase may enhance pain-related inflammatory responses, thereby increasing the risk of PHN.

4.3. Mediating role of immune cells in TN

In the causal relationships of TN, four blood cell phenotypes significantly influence the risk of TN through the mediation of five immune cell phenotypes. The perturbation response phenotype of neutrophils significantly increases the risk of TN through the expression of CD86 on granulocytes (β=0.373, P<0.001). CD86 is an important molecule involved in immune co-stimulation, and its increased expression in granulocytes may promote inflammatory signaling, thereby exacerbating pain[49, 50]. Conversely, the perturbation response phenotype of monocytes significantly reduces the risk of TN through double-positive T cells (CD4+CD8+) (β=-0.112, P=0.003). This result is consistent with the anti-inflammatory role of double-positive T cells[51, 52], indicating that these cells may reduce the incidence of TN by decreasing inflammation.

Immune cells play a key mediating role in the causal relationships between blood cell phenotypes and neuropathic pain. Specific immune cells, such as CD39+ regulatory T cells, HLA-DR+ CD4+ lymphocytes, and double-positive T cells, regulate inflammation and immune responses, thereby promoting or inhibiting the onset of conditions like PDPN, PHN, and TN. The study findings indicate that the mediating role of immune cells in neuropathic pain is complex and diverse. Further research into these immune mechanisms may provide new therapeutic targets for pain management.

This study has several notable advantages. First, we are the first to use Mendelian randomization (MR) methods to explore the causal effects of 91 blood cell phenotype-related characteristics and 731 immune cell phenotypes on the risk of neuropathic pain (PN), minimizing the impact of residual confounding factors. Second, we conducted an in-depth investigation into the mediating role of immune factors in the relationship between blood cell phenotypes and the risk of PN, providing valuable insights for future research on the pathogenesis of PN-related risk factors. Finally, we performed comprehensive sensitivity analyses to ensure the reliability of our results.

However, our study also has some limitations. On one hand, the sample size of the exposure is relatively small, which may affect the accuracy of the Mendelian randomization (MR) analysis. On the other hand, this study utilized publicly available genetic association data and blood cell phenotype information, which were obtained from different cohorts, potentially leading to data heterogeneity. Variations in age, sex, ethnicity, and other factors among participants in different studies may introduce confounding effects, thereby impacting the generalizability of our conclusions regarding the associations between neuropathic pain, blood cell phenotypes, and immune cells. Furthermore, this study explored the influence of blood cell phenotypes on neuropathic pain through the mediation of immune cells. However, the analysis of mediation effects is very complex, especially when multiple cell types and pathological processes are involved, limiting the precision of deriving causal chains. Although we employed two-sample MR analysis, there may still be some potential mediators or interactions that we overlooked.

This study systematically explored the potential causal roles of blood cell phenotypes and immune cells in neuropathic pain (including PDPN, PHN, and TN) using MR analysis methods. The results indicate that different types of PBC are significantly associated with the risk of developing neuropathic pain, with the perturbation responses of red blood cells, platelets, and neutrophils being closely linked to increased risks of PDPN, PHN, and TN. Additionally, certain phenotypic changes in eosinophils may play a protective role in reducing pain risk. The mediating role of immune cells between blood cells and neuropathic pain was also significantly demonstrated, with regulatory T cells (Tregs) and immune cells expressing markers such as HLA-DR, CD39, and CD86 showing key roles in pain regulation. These findings reveal the complex interactions between blood cells and the immune system in neuropathic pain, providing new insights into the pathophysiological mechanisms of neuropathic pain. However, these findings need to be further validated through broader clinical studies.

Ethics approval and consent to participate

Since this study relies entirely on publicly accessible aggregated data, no additional ethical approval or participant consent was necessary.

Consent for publication

Not applicable.

Availability of data and materials

The datasets analyzed during the present study are available as GWAS summary data. The immune cell traits (731 in total) can be accessed at GWAS MRC IEU, with the accession numbers Ebia-GCST0001391 to Ebi-a-GCST0002121. The summary statistics of 91 blood cell phenotypes (Study accession numbers: GCST90257015 to

GCST90257105) used in this article can be downloaded from the
NHGRI-EBI GWAS Catalog. The datasets for painful diabetic polyneuropathy (fnn-b-DM_POLYNEURO) can be accessed here. The datasets for post-herpetic neuralgia (fnngen_R9_G6_POSTZOST) are available here. The datasets for trigeminal neuralgia (fnngen_R10_G6_TRINEU) can be accessed here. These datasets were sourced from the FinnGen consortium's GWAS summary data, accessible at FinnGen.

Competing interests
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author contributions
CQW: Data curation, Software, Writing–original draft. ZT: Writing–original draft. LJ: Writing–review & editing. CDP: Writing–review & editing. GH: Methodology, Writing–original draft. CMB: Project administration, Supervision, Writing–review & editing.

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