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# **Single-cell RNA sequencing reveals heterogeneity of mucosa-associated invariant T cells in donor grafts and its diagnostic relevance in gastrointestinal graft-versus-host disease**

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## **Author contributions**

M.G. designed and performed flow cytometry and mouse experiment; analyzed the scRNA-seq, flow cytometry, and clinical data; and wrote the manuscript. S.L. assisted with scRNA-seq data analysis. K.Z., X.C. and H.G. participated in mouse experiment implementation and sample collection. Y.S., J.K., T.H. and Y.C. provided critical expertise and collected data. X.H., Y.C. and X.Z. provided experimental platform, guided the project and contributed to the manuscript. X.Z. designed the project and data analysis strategies, oversaw all raw data, and revised the manuscript.

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### **Disclosures**

The authors declare they have no competing interests.

### **Data sharing statement**

All data associated with this study are present in the paper or the Supplementary files. The data that support the findings of this study are openly available in The raw sequencing data generated by this project were deposited into the Genome Sequence Archive (<https://ngdc.cncb.ac.cn/gsa-human>) with accession number GSA - Human: HRA006712. At <https://ngdc.cncb.ac.cn/gsa-human>, reference number HRA006712.

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## ABSTRACT

Granulocyte colony-stimulating factor (G-CSF) enhances acute graft-versus-host disease (aGVHD) prophylaxis in allogeneic hematopoietic stem cell transplantation (allo-HSCT) by inducing T cell tolerance and altering graft cell composition. Previous studies have shown that the number of mucosa-associated invariant T (MAIT) cells in G-CSF induced graft was associated with a low incidence of gut aGVHD. However, the effect of G-CSF mobilization on MAIT cell and its role in MAIT-mediated protection against gut GVHD remain unclear. Here, using single-cell RNA sequencing (scRNA-seq), we found that the interaction of G-CSF with its receptor CSF3R enhances of immunosuppression and tissue repair functions of MAIT cells, contributing to the anti-gut aGVHD effect. The chemokine receptor CXCR6 was identified as potentially crucial for recruiting these functional MAIT cells to gut tissues. Besides, we simulated the dynamic distribution of MAIT cells from donor G-CSF mobilized peripheral blood stem cells (G-PBSCs) grafts in recipient mouse, and further confirmed that the circulating MAIT cells migrated into gut tissue in a CXCR6-CXCL16 dependent manner. To further validate these findings, we developed a flow cytometry panel that effectively predicts the gut aGVHD occurrence following allo-HSCT by analyzing the frequency and functional markers of MAIT cells in G-PBSCs. The predictive model shifts aGVHD prediction and intervention to the pre-transplant stage and offers a new strategy for the prevention and management of gut aGVHD in clinical practice.

**Keywords** Mucosa-associated invariant T cells; Granulocyte colony-stimulating factor; Gut acute graft-versus-host disease; Immunosuppression; Tissue repair; Chemotaxis

## INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an effective treatment for hematological malignancies. However, graft-versus-host disease (GVHD) remains a major obstacle, leading to transplantation failure and reduced overall survival. The overactivation of donor-derived T cells is recognized as a primary instigator of GVHD.<sup>1-2</sup> Granulocyte colony-stimulating factor (G-CSF) is widely used to mobilize hematopoietic stem/progenitor cells and exerts immunomodulatory effects in allo-HSCT, particularly in allo-HSCT strategies without T cell depletion. G-CSF promotes T-cell tolerance and alters graft cell composition, which can help to prevent GVHD.<sup>3-5</sup> In healthy donors, G-CSF administration induces a shift in T cells from a Th1 to Th2 phenotype while preserving the graft-versus-leukemia (GVL) effect.<sup>4-6</sup> Additionally, G-CSF modulates graft composition by promoting immune tolerance, suppressing NK cell activity, and expanding regulatory T cells.<sup>5-7</sup> However, its impact on unconventional T cells, particularly mucosal-associated invariant T (MAIT) cells, is poorly understood.

MAIT cells, a subset of multifunctional innate-like T cells, are abundant in human blood (1%-10% of T cells) and mucosal tissues, including the gut (10%), lungs, and liver (20%-40%).<sup>8-11</sup> A distinctive feature of MAIT cells is their recognition of the non-polymorphic MR1 molecule presenting riboflavin metabolite derivatives such as 5-OP-RU.<sup>12,13</sup> Once activated, MAIT cells exhibit both conventional effectors and tissue-reparative functions.<sup>14-17</sup> This dual role highlights their importance in protecting mucosal surfaces from microbial threats.<sup>17</sup> In mice, MAIT cells differentiate into T-bet<sup>+</sup> MAIT1 and ROR $\gamma$ t<sup>+</sup> MAIT17 subsets, which contribute to infection control and tissue repair, respectively.<sup>10,18-23</sup> Our previous work shows that MAIT cells in grafts are involved in suppressing gut aGVHD, potentially via interactions with specific gut microbiota. Furthermore, G-CSF mobilization enhances the expression of ROR $\gamma$ t in MAIT cells from donor peripheral blood stem cell grafts (PBSCs).<sup>24</sup> However, how G-CSF mobilization affects graft-derived MAIT cell function in recipients and their role in regulating gut GVHD is still unclear.

In adult human blood, MAIT cells express tissue-homing chemokine receptors such as CCR5, CCR6, CXCR6, and CCR9.<sup>14-17</sup> Increasing evidence suggests that chemotaxis is critical for MAIT cell-mediated antimicrobial and tissue repair.<sup>25,26</sup> Studies have shown that MAIT cells can be recruited from circulation to inflamed tissues, such as skin wounds.<sup>12</sup> In the context of allo-HSCT, the reconstruction of MAIT cells in recipients largely depends on their abundance in grafts, due to the pre-transplant myeloablative conditioning regimen.<sup>21,27,28</sup> Therefore, both the tissue migration capability and the abundance of MAIT cells in target tissues are crucial for mucosal protection and the prevention of gut aGVHD. However, the dynamics of MAIT cell distribution and their chemotactic heterogeneity during allo-HSCT are not well understood.

To investigate the role of G-CSF in the anti-gut aGVHD effects of MAIT cells, we integrated single-cell RNA sequencing (scRNA-seq) with *in vitro* and *in vivo* analyses. G-CSF-induced MAIT (G-MAIT) cells exhibited a transcriptional shift toward immunosuppressive and reparative profiles. In murine models, donor-derived G-MAIT cells showed dynamic tissue distribution and chemotactic targeting. These findings reveal a G-CSF-driven mechanism underlying MAIT cell – mediated protection against gut aGVHD and highlight their potential as biomarkers and therapeutic targets in allo-HSCT.

## **METHODS**

### **Human samples**

Peripheral blood (PB) samples were obtained from four healthy donors before and after G-CSF administration (filgrastim, 5 µg/kg/day for 5 days) for scRNA-seq (Table S1). For multiparameter flow cytometry (FCM), the PB of 16 healthy donors was collected before and after G-CSF administration. A clinical cohort of 80 G-CSF-mobilized PBSC grafts (G-PBSCs) was also analyzed (Table S2-S3) (see Supplementary Methods).

All procedures were approved by the Ethics Committee of Peking University People's Hospital (2018PHB222-01), and informed consent was obtained per the Declaration

of Helsinki.

### **Mucosa-associated invariant t cell isolation and single cell RNA analysis**

MAIT cells (CD3+CD161<sup>hi</sup>Vα7.2+) were isolated from PBMCs before and after G-CSF via fluorescence-activated cell sorting (FACS). scRNA-seq libraries were generated using 10x Genomics and sequenced on the Illumina NovaSeq 6000. Data were processed using Cell Ranger (v6.0.2) and analyzed with Seurat (v3.2.2)<sup>29</sup> in R. UMAP was used for dimensionality reduction and canonical markers for cluster annotation. Differential expression was analyzed using the Wilcoxon rank-sum test with Benjamini–Hochberg correction. Gene set enrichment analysis (GSEA) was performed using clusterProfiler (v4.10.0)<sup>30</sup> (see Supplementary Methods).

### **Signature scoring**

Gene set scores were calculated using Seurat's AddModuleScore. Gene sets included immunosuppression (61 genes),<sup>31,32</sup> cytotoxicity (*GZMA*, *GZMB*, *NKG7*, *IFNG*, *KLRD1*, *FGFBP2*, *GNLY*) and tissue repair genes (*VEGFB*, *VEGFA*, *IL17A*, *IL17RE*, *TGFB1*, *CCL3*, *FURIN*, *AREG*, *CSF1*, *TNF*)<sup>20</sup> (details in Table S4).

### **Flow cytometry**

Flow cytometry (FACS Canto II, BD Biosciences) assessed MAIT cell frequency and function. Antibodies included CD45, CD3, CD161, Vα7.2, CCR6, CXCR6, CD114, IL-17A, IL-4, IFN-γ (details in Table S5).

### **In vitro suppression and cytotoxicity assays**

PBMCs from donors were cultured with or without G-CSF (2μL/well, 24h), followed by sorting of MAIT and CD4+CD25- T cells. CFSE-labeled CD4+T cells were co-cultured with MAIT cells at 1:1, 2:1, 4:1 in RPMI-1640 with CD3/CD28 beads for 96h, followed by proliferation assessment via FCM. For cytotoxicity, G-CSF-treated or untreated MAIT cells were co-cultured with K562 cells (1:1) under IL-12/IL-18 and CD3/CD28 stimulation. Apoptosis was evaluated by Annexin V/7-AAD staining (see Supplementary Methods).

### **GVHD mouse model and CXCL16 blockade**

NOD-SCID-IL2Rγ<sup>-/-</sup> (NSG) mice received 180 cGy irradiation followed by 2×10<sup>6</sup>

human G-PBSCs to establish xenogeneic GVHD. Mice received intraperitoneal injections of anti-CXCL16 antibody (100µg) or PBS twice in the first week post-transplant. Survival, body weight, and GVHD scores (0–6) were monitored. Intestinal tissues were analyzed by immunofluorescence for CD3, CD4, CD8, and CD161 (see Supplementary Methods).

### **Diagnosis of acute GVHD and predictive score calculation**

Gut aGVHD was diagnosed clinically and histologically per established criteria.<sup>33,34</sup> MAIT cells and their subsets (CD114+, IL-17A+, IL-4+, CXCR6+) were quantified by FCM. Bootstrap resampling established median thresholds for each subset. Binary scores (1 or 0) per subset were summed into a composite predictive score (range 0–5) (see Supplementary Methods).

### **Statistical analyses**

Data are presented as mean±SEM. Continuous variables were compared using the Mann–Whitney U test or one-way ANOVA, as appropriate. Cumulative incidences were compared using Gray's test. P-values <0.05 were considered significant. Analyses were performed using GraphPad Prism 9.0 and SPSS v23 (Chicago, IL, USA).

## **RESULTS**

### **G-CSF induces MAIT cells to enhance immunosuppression and tissue repair**

We first assessed the impact of G-CSF on MAIT cells via FCM (Fig.S1A). No significant change in MAIT cell frequency was observed in PB of donors before and after G-CSF mobilization (Fig.1A). Considering IFN-γ, IL-4, and IL-17A as hallmark effector cytokines of Th1, Th2, and Th17 cells, respectively, we evaluated their expression in MAIT cells. G-MAIT cells exhibited a marked increase in IL-4 expression relative to IFN-γ and IL-17A (Fig.1B and Fig.S1B), indicating G-CSF-induced type 2 polarization and enhanced immune tolerance, consistent with previous reports of G-CSF-mediated T cell polarization.<sup>2</sup>

To better characterize G-MAIT cells, we collected paired PB samples from four healthy donors before and after 5-day G-CSF treatment. Using FACS, we isolated

MAIT cells for subsequent scRNA-seq (Fig.1C). After quality control, 80709 MAIT cells were clustered into 14 clusters reflecting functional heterogeneity (Fig.1D, Table S6). Notably, Cluster 7 was enriched for cytotoxicity-related genes (*NKG7*, *IFNG*, *GZMB*), Cluster 5 expressed naïve markers (*CCR7*, *SELL*, *LEF1*), and Cluster 13 displayed a proliferative signature (*MKI67*, *STMN1*) (Fig.S1C, Table S6).

Contrary to murine MAIT cell subsets, human MAIT1 and MAIT17 cells demonstrated a mixed expression of *TBX21* and *RORC* at transcript and protein levels (Fig. S1D), corroborating prior studies.<sup>8,9</sup> Although distinct Th1, Th2 and Th17 phenotypes were not isolated, G-MAIT cells showed upregulation of genes related to Th2 differentiation (IL-4 and IL-13 signaling) (Fig.1E) and a higher G-CSF score (Fig.1F) compared to MAIT cells without G-CSF administration, This suggested that G-CSF induces IL-4-driven polarization and enhances immune tolerance.

Functional analyses revealed that G-MAIT cells exhibited enhanced immunosuppressive and tissue repair gene signatures, accompanied by reduced cytotoxic profiles (Fig.1G). G-CSF also enhanced expression of migration and tissue residency markers (Fig.S1E), supporting their role in tissue repair. Furthermore, G-MAIT cells showed decreased expression of cytotoxic genes (*NKG7*, *GNLY*, *FGFBP2*, *GZMA*), increased expression of naïve-associated genes (*SELL*, *LEF1*) (Fig.S1F). Immune checkpoint molecules (*PDCD1*, *TIGIT*, *LAG3*, *CTLA4*), proliferation markers (*MKI67*, *CDK1*), and co-stimulatory molecules (*CD28*, *TNFRSF9*, *TNFRSF14*) were also upregulated, though FCM results were not statistically significant (Fig.S2A).

To validate the immunoregulatory capacity of G-MAIT cells, *in vitro* co-culture assays with CD4<sup>+</sup> T cells were performed. G-MAIT cells exhibited significantly stronger suppression of CD4<sup>+</sup> T cell proliferation compared to controls at MAIT/CD4 ratios of 1:1 (P = 0.023) and 4:1 (P = 0.043) (Fig.1H). Cytotoxicity against K562 cells under IL-12/IL-18 or CD3/CD28 stimulation was comparable between G-MAIT and control MAIT cells (Fig.S2B). These results demonstrate that G-CSF enhances MAIT cell immunoregulatory capacity without impairing cytotoxic function.

### **G-CSF-CSF3R interaction mediates the functions of MAIT cells**

To elucidate how G-CSF modulates MAIT cell function, we performed differentially expressed genes (DEGs) analysis, which revealed upregulation of genes associated with immunosuppression (*NFKBIA*, *ZFP36*, *DUSP2*),<sup>31,38,39</sup> Th2 developmental (*JUNB* and *MT2A*)<sup>31</sup> and IL-17 signaling (*JUN* and *FOS*), as well as tissue residency (*CD69*, *H3F3B*, *JUN*, *TNFAIP3*) following G-CSF mobilization (Fig.2A and Fig.S3A) (details in Table S7).<sup>40,41</sup> Notably, these genes were enriched in Cluster 12, characterized by high expression of *CSF3R* (G-CSF receptor), which the proportion and G-CSF score of Cluster 12 significantly increased after G-CSF mobilization (Fig.2B and Fig.S3B). These findings suggested that G-CSF could mediate MAIT cell function through *CSF3R* of Cluster 12.

Previous studies have shown that G-CSF, by binding to *G-CSFR*, directly regulate downstream targeted genes related to anti-inflammation and immunosuppression (*ZFP36* and *DUSP2*).<sup>31</sup> Consistently, *CSF3R* and its targets were upregulated after G-CSF stimulation (Fig.2C). Cluster 12 exhibited a stronger immunosuppressive signature than other clusters (Fig.S3C), suggesting that its hyporesponsiveness may result from the direct G-CSF–*CSF3R* signaling. GSEA of Cluster 12 showed the enrichment of IL-17 and TNF signaling pathway related to tissue repair (Fig.2D and Fig.S3D), supporting that Cluster 12 may be involved in tissue repair of G-MAIT cells. Additionally, downregulation of the Graft-versus-host disease pathway (Fig. 2D, S3D) supports a role for the G-CSF–*CSF3R* axis in Cluster 12 in mediating the anti-GVHD effects of G-MAIT cells.

FCM confirmed the presence of CD114+ MAIT cell subset (*CD114* encoded by *CSF3R*), which expanded after G-CSF induction (Fig.2E,  $P=0.048$ ). In a clinical G-PBSCs cohort, CD114 expression on G-MAIT cells was significantly lower in donors whose recipients developed gut aGVHD compared to those without aGVHD or with skin aGVHD (Fig.2F), further underscoring a potential role of CD114+ G-MAIT cells against gut GVHD.

### **Functional heterogeneity of G-MAIT cells correlates with gut aGVHD outcomes**

To investigate whether the functional states of G-MAIT cells influence gut aGVHD development, we stratified G-CSF-treated scRNA-seq samples by whether recipients developed gut aGVHD post-transplant (DEGs for details, see Table S8). G-MAIT cells from the no gut aGVHD group exhibited higher expression of *CSF3R* and its target genes (Fig.3A), indicating donor heterogeneity in G-MAIT cells response to G-CSF. They also exhibited enhanced immunosuppressive and tissue repair signatures compared to the gut aGVHD group (Fig. 3B), with increased expression of pro-repair mediators<sup>18,20</sup> including *TNF*, *FURIN*, *TGFB1*, *AREG* and *IL-17A* (Fig.S4A), along with enhanced tissue residency and migration signatures (Fig.3C). These findings imply that, beyond immunosuppression, the enhanced tissue repair, migration, and residency capabilities of G-MAIT cells are critical for maintaining intestinal mucosa integrity and preventing gut aGVHD.

Key markers associated with tissue residency, repair, immune tolerance, and cytotoxicity-CD69, IL-17A, IL-4, and IFN- $\gamma$ -were analyzed by flow cytometry in a clinical G-PBSCs cohort (Fig.3D-E). Results confirmed lower IL-4 and IL-17A expression in G-MAIT cells from donors whose recipients developed gut aGVHD, while other effector factors showed no significant differences (Fig.3E). These results further highlighted the importance of immunosuppressive and tissue repair functions of G-MAIT cells in mediating protection against gut aGVHD.

Given that tissue repair by circulating MAIT cells depends on chemokine receptor-mediated migration, we analyzed chemokine signaling and receptor expression. Chemokine signaling pathways were significantly downregulated in the gut aGVHD group (Fig.3F). To rigorously evaluate this, we analyzed expression of chemokine receptor genes in G-MAIT cells, previously used to evaluate MAIT chemotaxis.<sup>12,17,41</sup> scRNA-seq revealed distinct receptor profiles across MAIT clusters, reflecting functional heterogeneity. For example, *CXCR3* was enriched in cytotoxic Cluster 7, *CXCR6* in Cluster 3 and 6, *CCR7* in naïve cells, and *CCR1/CCR2* in Cluster 12 (Fig.S4B). The heterogeneity suggested differential migration, distribution and function among MAIT subsets under various microenvironment or pathological

conditions. Comparing no gut aGVHD and gut aGVHD groups (Fig.3G), G-MAIT cells from the no gut aGVHD group showed higher *CCR1*, *CCR5*, *CCR7*, *CXCR3*, and *CXCR6* expression, while *CCR2* and *CCR6* were relatively upregulated in the gut aGVHD group (Fig.3G). FCM validation confirmed significantly reduced expression of *CXCR6* and *CCR7* in the gut aGVHD group, with no significant differences for other receptors (Fig.3H and Fig.S4C). Overall, above results indicated that heterogeneous expression of key functional mediators of G-MAIT cells could be associated with the occurrence of gut aGVHD.

#### **G-MAIT cells exhibit gut-specific chemotaxis and anti-gut aGVHD effects *in vivo***

To assess the tissue-homing behavior of G-MAIT cells after allo-HSCT, we used a humanized NSG mouse model (Fig.4A). Engraftment of human white blood cell (WBC) was detected from PB samples of mice at 8-14 days after transplantation. With GVHD progressed, MAIT cell abundance increased within organ tissues, particularly in the intestine, as compared to PB and bone marrow (Fig.4B-D). On Day 14 after transplantation, mice were divided into two groups based on their GVHD Score ( $\geq 3$  or  $< 3$ ). Notably, mice with lower GVHD scores exhibited higher intestinal MAIT cell infiltration (Fig.4E), indicating that MAIT cells preferentially migrated to the gut and contributed to mitigating gut aGVHD. These findings highlight the gut-directed chemotactic capacity of G-MAIT cells as a key mechanism underlying their protective effect.

#### **CXCR6 is required for G-MAIT cell recruitment to gut tissue**

*CXCR6*, a key receptor for liver-intestinal targeting,<sup>42</sup> emerged as the most differentially expressed chemotactic receptor gene between no gut aGVHD and gut aGVHD group (Fig.3G-H). We therefore focused on its function in intestinal chemotaxis. To assess its role in intestinal homing, we blocked *CXCR6*-*CXCL16* interaction using *CXCL16* blocking antibody in NSG mice (Fig.5A). *CXCL16* blockade led to a marked reduction in gut-infiltrating MAIT cells post-transplantation, with minimal impact on PB, bone marrow, or other tissues (Fig.5B), indicating that *CXCR6*-*CXCL16* was critical for gut recruitment of G-MAIT cells. Additionally,

mice in the Anti-CXCL16 group showed more severe GVHD symptoms such as hair loss, visceral congestion and had higher GVHD scores, especially on Day 16 after transplantation (Fig.5C). Histological examination confirmed increased cellular infiltration and mucosal disruption in the intestines of anti-CXCL16-treated mice (Fig.5D), consistent with aggravated gut inflammation.

Immunofluorescence analysis further demonstrated impaired intestinal localization of CD161<sup>+</sup> T cells upon CXCL16 blockade, whereas CD3<sup>+</sup>CD161<sup>+</sup>-T cells were less affected (Fig.S5A-5B). Previous studies have confirmed that circulating MAIT cells are predominantly CD8<sup>+</sup> (approximately 70%) and double-negative (DN, approximately 15%).<sup>17</sup> However, FCM results showed that gut-resident MAIT cells were predominantly CD4<sup>+</sup>CD8<sup>-</sup>, followed by CD8<sup>+</sup> cells (Fig.S5C). Notably, CXCL16 blockade significantly reduced both the frequency and density of intestinal CD3<sup>+</sup>CD161<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> T cells, with no apparent changes observed in the CD8<sup>+</sup> and CD4<sup>+</sup>CD8<sup>-</sup> MAIT subsets (Fig.5E-5F and Fig.S5D). Despite these changes, overall survival was not significantly different between groups (Fig.5G). Collectively, these results suggested that G-MAIT cell recruitment to the gut after allo-HSCT depends on the CXCR6-CXCL16 axis.

### **A predictive MAIT cell-based panel for gut aGVHD**

scRNA-seq analysis, supported by in vitro and in vivo experiments, identified a gene set (*CSF3R*, *IL17A*, *IL4*, and *CXCR6*)-potentially involved in mechanism of anti-gut aGVHD (Fig.6A). Gene set scoring revealed significantly higher expression in G-MAIT cells from the no gut aGVHD group, suggesting its predictive potential (Fig.6A).

To validate the clinical translational potential of these findings, we analyzed donor G-PBSCs. Consistent with prior work<sup>21</sup>, gut aGVHD onset was associated with a lower frequency of G-MAIT cells, whereas skin aGVHD showed no such correlation (Fig.6B-C). This result further confirmed that the frequency of MAIT cells in G-PBSCs can specifically predict the onset of gut aGVHD. Other T cell subsets, including conventional CD4<sup>+</sup>, CD8<sup>+</sup> T cell and non-conventional  $\gamma\delta$  T cells, found no

significant differences between the gut aGVHD and other groups (Fig.6B and Fig.S6A). Further analyses by aGVHD grades showed no significant differences in frequencies of total T (CD3+T cells) and T cell subsets ( $\gamma\delta$  T, CD4+, CD8+ T and MAIT cells) (Fig.S6B) across I, II–IV and no aGVHD groups, nor in expression of functional markers CD114, CXCR6, IL-17A and IL-4 (Fig.S6C). These data suggest that the reduction in MAIT cells is more pronounced in gut involvement rather than overall GVHD severity, supporting their potential role as immunological correlates of gut aGVHD.

To enhance predictive power, we developed a composite score integrating MAIT cell frequency and expression of functional markers (CD114, IL-17A, IL-4, CXCR6) (Fig.6D). This score was significantly lower in patients with gut aGVHD than in those with no or skin aGVHD (Fig.6D). Importantly, low-score individuals exhibited a higher incidence of gut aGVHD, whereas no association was observed with skin aGVHD (Fig.6E). The score was not associated with aGVHD severity grade (Fig. S6D), nor did the incidence of grade II–IV aGVHD differ between high- and low-score groups (Fig. S6E), indicating its specificity for gut aGVHD prediction.

Notably, this predictive panel outperformed MAIT cell frequency alone in predicting gut aGVHD, achieving a sensitivity of 75% and specificity of 93% (Fig.6F). In univariate analysis, low score, EBV infection, and recipient age were risk factors for gut aGVHD, while multivariate analysis identified the low predictive score as an independent predictor (Fig.6G). Overall, these results support a clinically applicable, FCM-based predictive panel incorporating both MAIT cell frequency and functional phenotype as a specific biomarker for gut aGVHD.

## DISCUSSION

This study reveals that G-MAIT cells suppress gut aGVHD by enhancing immunoregulatory and tissue repair functions via the G-CSF–CSF3R axis. The gut homing of these MAIT cells is mediated by the CXCR6–CXCL16 chemokine pathway. We also developed a flow cytometry-based MAIT cell score, which integrates both cell frequency and phenotype in grafts, to predict gut aGVHD risk pre-

transplant, providing clinical translational value.

Previous studies have shown that peripheral MAIT cells in both mice and humans exhibit a broad range of phenotypes, spanning homeostatic, effector, cycling, regulatory, and exhausted states.<sup>8,31,43,44</sup> Consistent with the previous studies,<sup>8,44,45</sup> our data showed most MAIT cells in human PB exhibit a mixed MAIT1/MAIT17 transcriptome, co-expressing genes associated with tissue repair, cytotoxicity, type1/17 features. This mixed differentiation program appears to be a conserved feature of innate-like T cells across evolution.<sup>44</sup> A key finding of our study is the identification of a G-CSF–induced MAIT cell subset (Cluster 12) characterized by CSF3R expression. These G-MAIT cells showed high expression of genes involved in immunosuppressive and IL-17 signaling pathways, along with enrichment of *CSF3R* target genes, suggesting that G-CSF–CSF3R signaling promotes immunosuppressive and tissue-reparative functions in MAIT cells. This is further supported by the enrichment of transcripts encoding *ZFP36*, *ZFP36L1*, and *ZFP36L2* in Cluster 12, which are known to regulate the stability of mRNA for cytokines and immune mediators.<sup>12,46</sup>

MAIT cells are enriched in mucosal tissues and play a key role in mucosal immunity through their unique activation by microbial riboflavin metabolites and their ability to initiate a tissue repair program upon TCR trigger. The reparative function of MAIT cells has been primarily studied in mucosal tissues such as the skin,<sup>12</sup> lungs<sup>48</sup> and colon.<sup>49</sup> Our data demonstrate that circulating MAIT cells also express tissue repair signatures, which are further upregulated following G-CSF mobilization. We hypothesize that this subset, predominantly MAIT17 effector memory cells with a preactivated repair program, can be rapidly recruited to sites of mucosal injury or inflammation, where local environmental cues trigger their reparative functions. This hypothesis is supported by a recent study on skin wound healing using the Kaede photoconvertible mouse, which showed that MAIT cells accumulating in skin wounds originate from the circulation, including lymphoid organs.<sup>12</sup> In addition, the number of MAIT cells in PB of patients was significantly

reduced during inflammation or GVHD,<sup>21,49</sup> further indicating their recruitment from the blood to the pathological sites.

MAIT cells play a critical role in maintaining intestinal homeostasis through their ability to sense microbial-derived riboflavin metabolites and initiate tissue repair responses.<sup>18,47,49</sup> Inflammatory conditions can disrupt the anaerobic environment of the gut, resulting in an increased synthesis of MAIT cell ligands by the microbiota. These MAIT ligands can penetrate the intestinal barrier and activate MAIT cells across various tissues, thereby triggering epithelial repair and enhancing the protection against intestinal inflammation.<sup>49</sup> In the context of allo-HSCT with myeloablative conditioning, our previous work showed that higher levels of MAIT cells in grafts correlate with a reduced incidence of gut aGVHD.<sup>21</sup> This protective effect is likely mediated by both immunoregulatory functions and microbiota-driven activation, resulting in elevated IL-17A production—an essential cytokine for intestinal repair and barrier maintenance.<sup>15, 18, 47,48</sup> The gut-selective protection of MAIT cells contrasts with their limited impact on skin aGVHD, potentially reflecting differences in MAIT cell abundance (2–10% vs. 0.5–2% of CD3+T cells) and microbial stimulation between tissues.<sup>17</sup> MAIT cell-mediated repair is closely linked to MR1-dependent activation. Compared with MR1<sup>+/−</sup> NOD mice, increased intestinal permeability was observed in MR1<sup>−/−</sup> NOD mice.<sup>50</sup> Consequently, the immunosuppression and tissue repair functions triggered by the microbial environment likely constitute key mechanisms through which MAIT cells exert their anti-gut aGVHD effect.

CXCR6 has been described to favor recruitment of MAIT cells to pathological tissue sites and is necessary for complete MAIT cell maturation in the thymus.<sup>12,48,50</sup> This mechanism is likely shared for MAIT cell recruitment to other organs, CXCR6-CXCL16 drives MAIT cell accumulation into the skin, lung and kidney.<sup>12,26,41</sup> Furthermore, CXCR6 is enriched in the MAIT17 subset, suggesting selective recruitment from circulation rather than proliferation in situ.

Notably, heterogeneity among MAIT cells in G-PBSCs from different donors was observed. MAIT cells in G-PBSCs with gut aGVHD recipients exhibited reduced immunosuppressive and tissue repair features, as well as lower CXCR6 expression. Based on available evidence, the mechanism through which G-MAIT cells provide resistance against gut aGVHD relies on two key factors. Firstly, it depends on the absolute number of MAIT cells in the gut tissues, which is influenced by both the number of MAIT cells transfused from G-PBSCs to the recipient,<sup>21</sup> as well as the chemotactic ability of circulating MAIT cells to migrate into the gut tissues. Secondly, it relies on the immunosuppressive and tissue repair capabilities of MAIT cells within G-PBSCs, which is modulated by G-CSF responsiveness via CSF3R expression. Thus, the immunosuppression, tissue repair, and chemotactic ability exhibited by G-MAIT cells are key to exerting its anti-gut aGVHD effect.

Of note, discrepancies exist across studies regarding the association between MAIT cells and aGVHD risk. While some have reported that higher MAIT cell abundance correlates with increased aGVHD risk,<sup>51,52</sup> others, including our own<sup>21</sup> and *Bhattacharyya et al.*,<sup>28</sup> have found that early MAIT cell reconstitution is associated with reduced aGVHD incidence. Several factors may contribute to these divergent findings. First, the dynamics of immune reconstitution could influence outcomes: although rapid MAIT cell recovery may indicate effective immune reconstitution, it could also reflect a heightened overall immune activation state, potentially promoting GVHD. Second, infections or endotoxemia may independently activate and expand MAIT cells and other effector T cells while concurrently triggering GVHD. Third, technical variations in MAIT cell identification and subset analysis across studies may account for inconsistent results. These discrepancies highlight the need for future studies that incorporate standardized MAIT cell definitions, synchronized assessments of peripheral and tissue-resident MAIT cells, and integration of single-cell transcriptomic and TCR sequencing analyses to more precisely evaluate the functional quality and reconstitution patterns of MAIT cells.

We have developed an innovative flow cytometry panel that integrates both the

frequency and phenotypic heterogeneity of MAIT cells in G-PBSCs, enabling accurate pre-transplant prediction of gut aGVHD. This panel significantly outperforms the approach that rely solely on MAIT cell frequency. In contrast to most current methods, which are applied post-transplant and require multiple time-point samples affected by numerous clinical factors, our model allows risk assessment using a single pre-transplant graft sample. This strategy offers practical advantages, including ease of sample collection, operational simplicity, and cost-effectiveness, making it well-suited for clinical application. Nonetheless, further validation with larger sample sizes is necessary, along with comparative evaluations of its accuracy and effectiveness against existing aGVHD prediction methods.

In implementing this model, we employed the widely used CD3+CD161<sup>hi</sup>Vα7.2+ gating strategy to identify MAIT cells, given its practicality and broad application in previous studies. However, CD161 expression can be downregulated under certain physiological and pathological conditions,<sup>53</sup> such as activation or infection, potentially leading to an underestimation of MAIT cell frequencies. While MR1 tetramer staining offers greater specificity and is not affected by activation status, its limited accessibility hinders its routine use in large-scale clinical settings. Therefore, our use of CD161-based gating reflects a balance between specificity and feasibility. We acknowledge this methodological limitation and have interpreted our findings with appropriate caution, especially in contexts where MAIT cell activation may vary.

## **Conclusions**

Our findings identify a novel G-CSF–responsive MAIT cell subset in G-PBSCs that suppresses gut aGVHD via enhanced immunoregulation and tissue repair. CXCR6-mediated trafficking to the gut and CSF3R-driven functional regulation are critical to this effect. A graft MAIT cell score incorporating CD114, IL-17A, IL-4, and CXCR6 provides a promising tool for pre-transplant risk stratification. These insights pave the way for MAIT cell–based interventions to prevent gut aGVHD and improve transplant outcomes.

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### Figure Legends:

**Fig.1 Characteristics of mucosa-associated invariant T (MAIT) cells before and after granulocyte colony-stimulating factor (G-CSF) administration.** (A) Flow cytometric analysis showing MAIT cell frequency in donor peripheral blood stem cells (PBSCs) before and after G-CSF-mobilization. (B) Flow cytometric analysis showing the expression of IL-4 ( $P=0.041$ ), IFN- $\gamma$  and IL-17A in MAIT cells from donor PBSCs ( $n=16$ ) before and after G-CSF mobilization. (C) Workflow showing the processing of MAIT cells of eight PB samples from 4 healthy donors before and after 5-day G-CSF treatment for scRNA-seq. (D) Uniform manifold approximation and projection (UMAP) visualization of 14 MAIT cell clusters identified in human PB based on scRNA-seq. Each dot represents a single cell, with colors indicating different clusters. (E) VlnPlot showing the expression of genes related to IL-4/IL-13 pathway before and after G-CSF mobilization. (F) Violin plot showing the G-CSF score of MAIT cells before and after G-CSF mobilization (\*\*\*\* $P$  value  $< 0.0001$ ). (G) Dot plot showing functional signatures of MAIT cells before and after G-CSF mobilization. (H) Representative flow cytometry results showing the immunosuppressive effect of MAIT cells on CD4 $^{+}$ T cells under C-CSF mobilization. MAIT and CD4 $^{+}$  T cells were isolated from the mobilized and unmobilized PB of three healthy donors. 5,6-carboxy-fluorescein diacetate succinimidyl ester (CFSE)-labeled CD4 $^{+}$  T cells were stimulated with CD3/CD28 beads and co-cultured with MAIT cells at ratios of 1:1, 2:1, 4:1 for 4 days.

**Fig.2 G-CSF-CSF3R interaction mediates MAIT cell functions.** (A) Dot plot showing the distribution of immune regulation-related genes among the Top 50 DEGs in MAIT cell clusters. (B) Violin Box plot showing the distribution of *CSF3R* expression and G-CSF score across different clusters. (C) Dot plot showing the expression of *CSF3R* and its target genes in MAIT cells. (D) Bar plots from gene set enrichment analysis (GSEA) analysis showing the major enriched terms of DEGs before (left) and after (right) G-CSF administration. (E) Flow cytometric analysis of CD114 expression in donor MAIT cells from PB before and after G-CSF mobilization. (F) Flow cytometric analysis showing CD114 expression of MAIT cells in G-PBSCs (G-MAIT) between groups of gut aGVHD ( $n=9$ ), skin aGVHD ( $n=13$ ) and no aGVHD ( $n=55$ ) (\* $P$  =

0.042;\*\*\*\* $P < 0.0001$ ).

**Fig.3 G-CSF-induced MAIT cells contribute to anti-gut aGVHD effects.** (A) Dot plot showing the expression of *CSF3R* and its target genes in G-MAIT cells between groups of gut aGVHD (n=1) and no gut aGVHD (n=3). (B) Dot plot illustrating the functional signatures of G-MAIT cells between the two groups. (C) Violin box plot showing the expression of migration- and tissue residency-related signatures between the two groups (\*\*\*\* $P < 0.0001$ ). (D) Flow cytometric analysis showing CD69 expression in G-MAIT cells between groups of gut aGVHD (n=9), skin aGVHD (n=13) and no aGVHD (n=55). (E) Flow cytometric analysis showing IL-4, IL-17A and IFN- $\gamma$  expression in G-MAIT cells among the three groups (\* $P < 0.05$ ;\*\*\* $P < 0.001$ ;\*\*\*\* $P < 0.0001$ ). (F) GSEA plots showing downregulation of the chemokine signaling pathway in G-MAIT cells from the no gut aGVHD group compared to the gut aGVHD group. (G) Dot plot showing chemokine receptor expression in G-MAIT cells between gut aGVHD and no gut aGVHD group. (H) Flow cytometric analysis showing chemokine receptor expression in G-MAIT cells between groups of gut aGVHD (n=9), skin aGVHD (n=13) and no aGVHD (n=55) (\* $P < 0.05$ ).

**Fig.4 Specific chemotactic migration of MAIT cells from G-PBSCs to the gut.** (A) Protocol for establishing a GVHD model in NSG mice by injecting human G-PBSCs. (B) Gating strategy for identifying human MAIT cells in NSG mice. (C) Flow cytometric analysis showing the dynamic distribution of human MAIT cells after transplantation in PB, bone marrow (BM), gut, liver and lung tissues of NSG mice. (D) Line graphs showing the dynamic changes in human MAIT cells post-transplantation in PB, BM, lung, gut and liver tissues of NSG mice. (E) Flow cytometric analysis showing the frequency of MAIT cells in gut tissues between mice with high GVHD score (GVHD Score $\geq 3$ ) and low GVHD (GVHD Score $< 3$ ) score on day 14 post-HSCT.

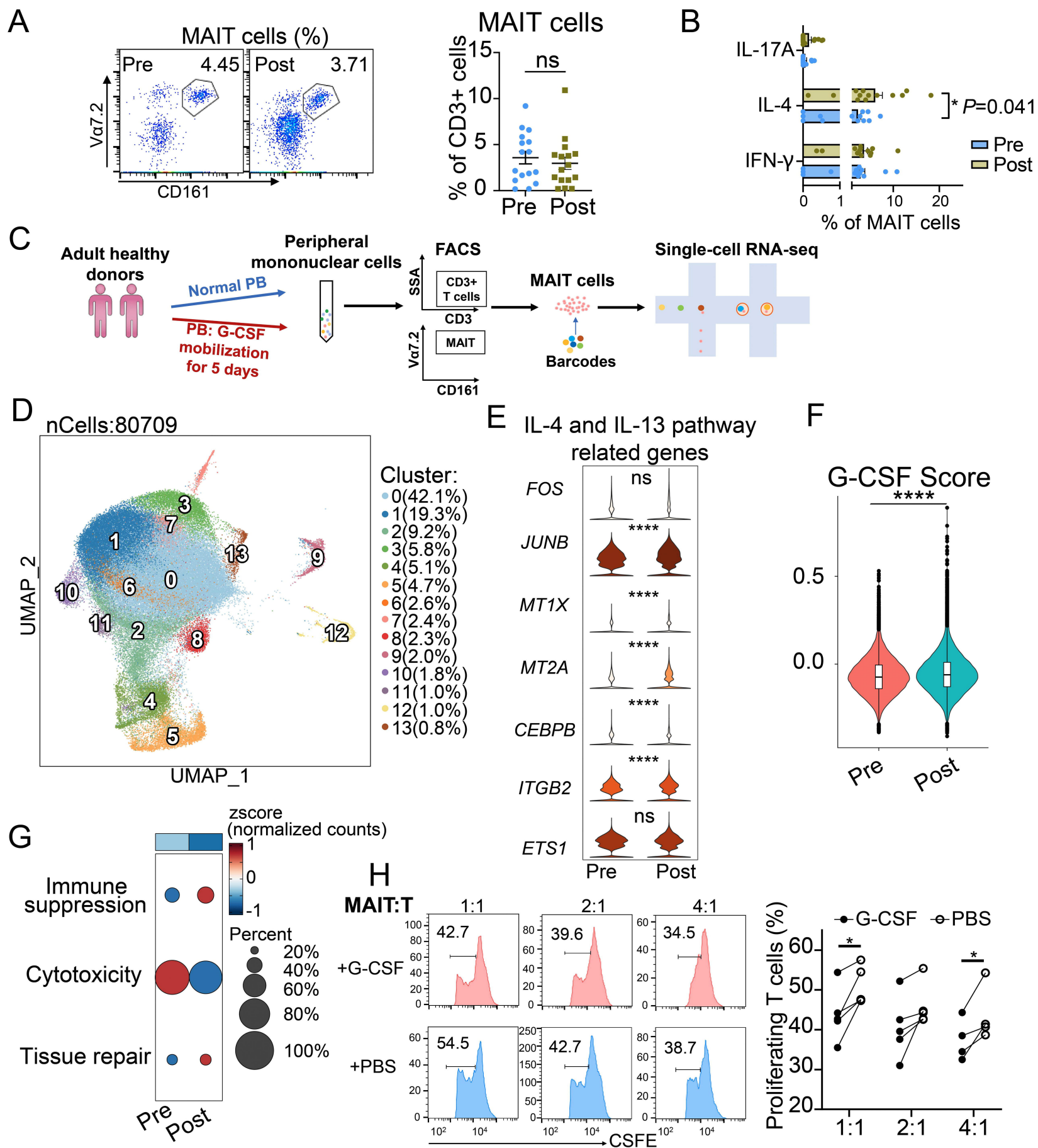
**Fig.5 CXCR6 is necessary for G-MAIT cell recruitment to gut tissue.** (A) Protocol for the establishment of CXCL16 blocking in NSG mice through injecting human G-PBSCs. (B) Representative flow cytometry showing MAIT cell frequencies in the gut of control and anti-CXCL16 treatment groups (left), and frequencies of human MAIT cells from PB, BM, gut, liver

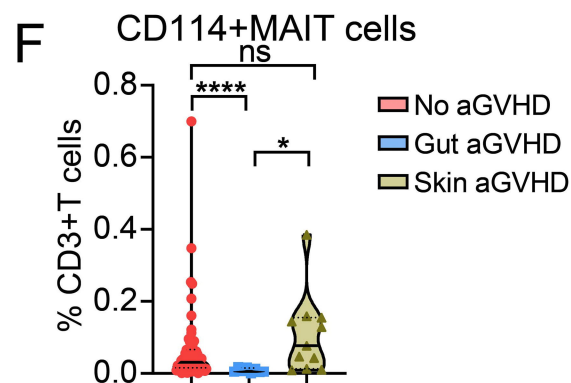
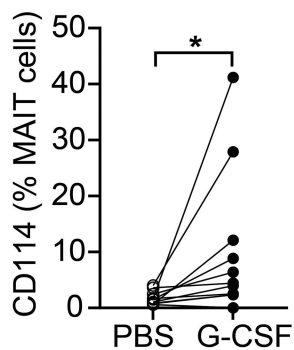
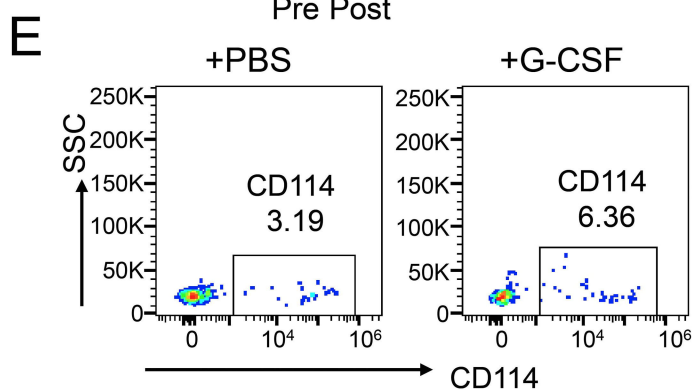
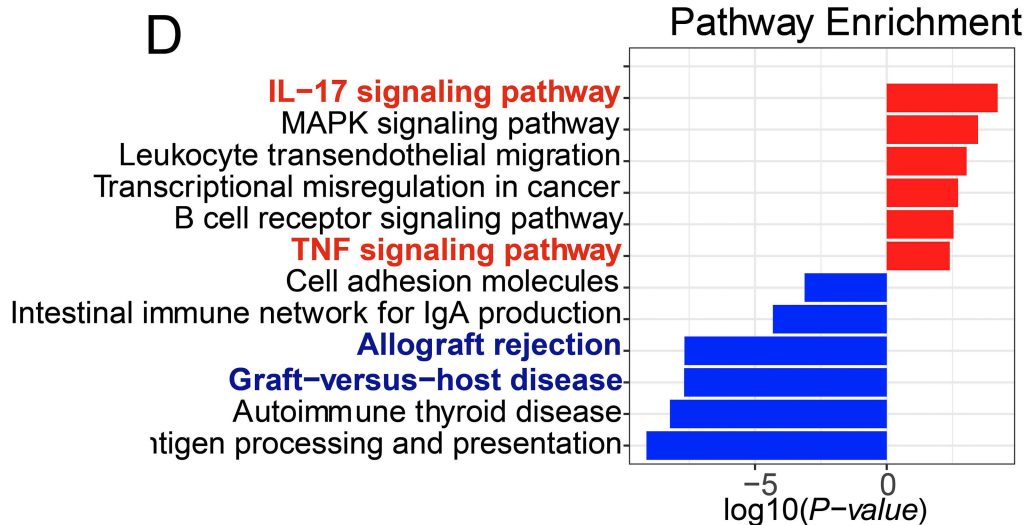
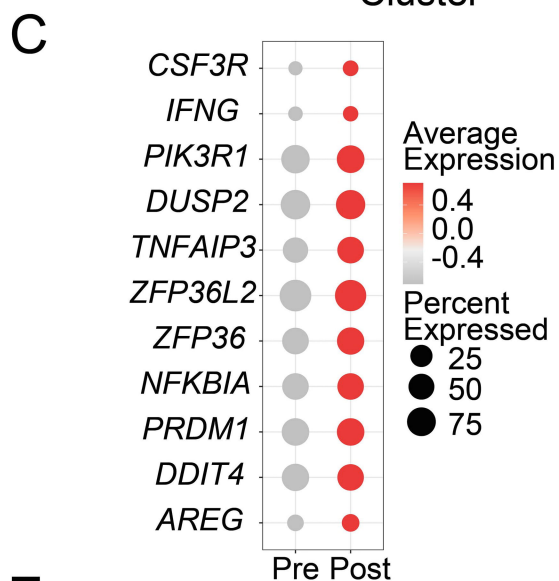
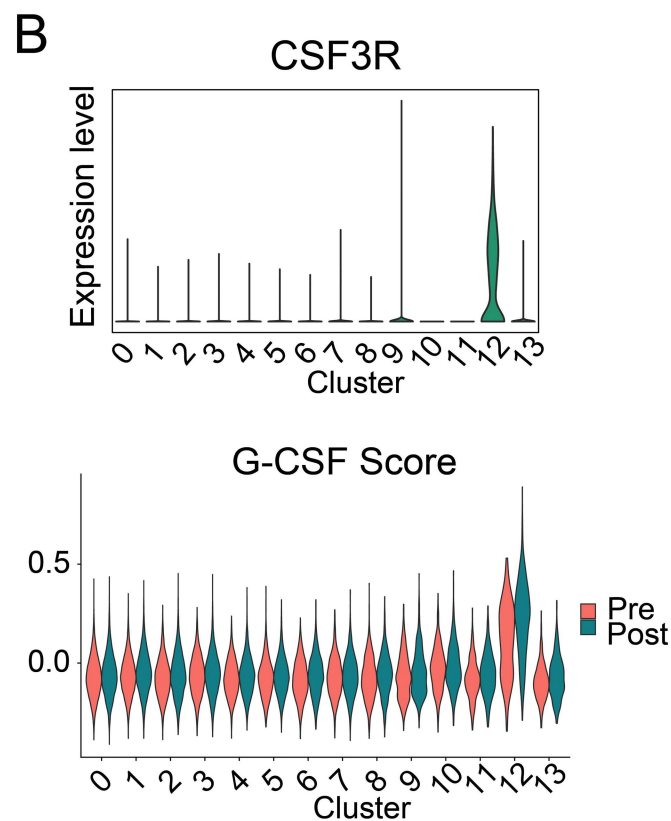
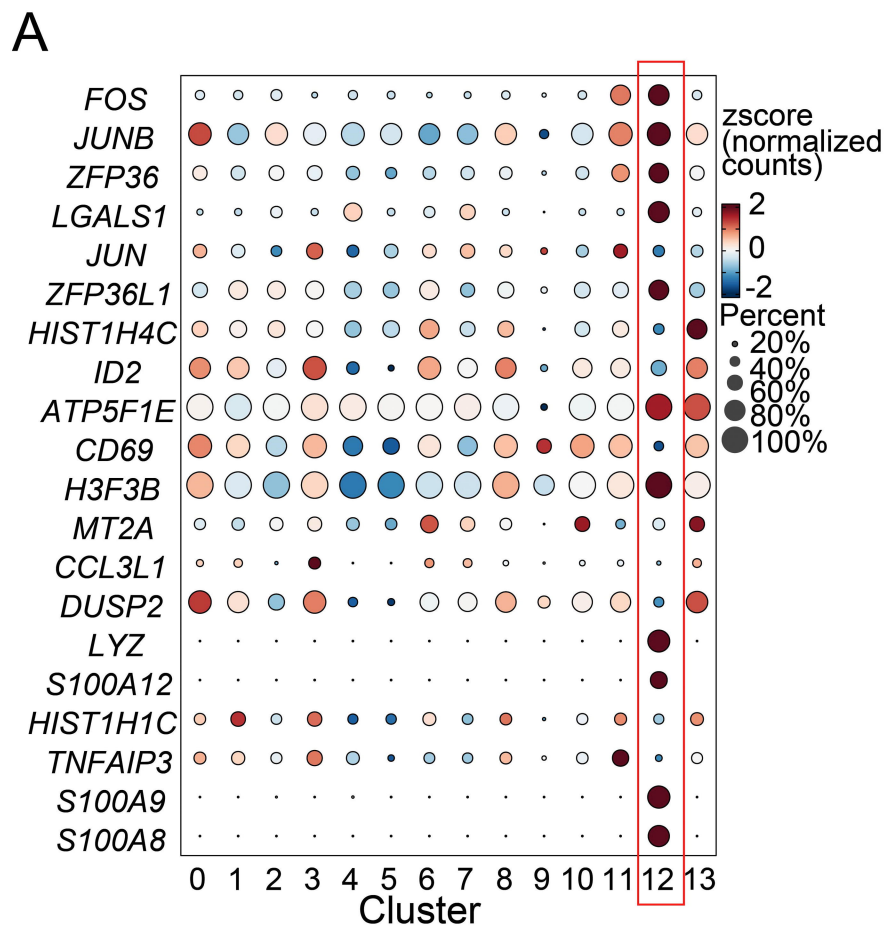
and lung tissues of NSG mice (right). **(C)** GVHD-related symptoms in control and anti-CXCL16 groups post-transplantation (left), and flow cytometric analysis of GVHD scores on day 16 and Day 18 post-HSCT (right). **(D)** HE staining showing representative histological features of intestinal tissues harvested from mice at the study endpoint post-transplantation. **(E) and (F)** Multiplex immunofluorescence analysis revealing the target cell populations in intestinal tissues at the study endpoint, comparing control and anti-CXCL16 groups. **(G)** Survival curve comparing control and anti-CXCL16 groups.

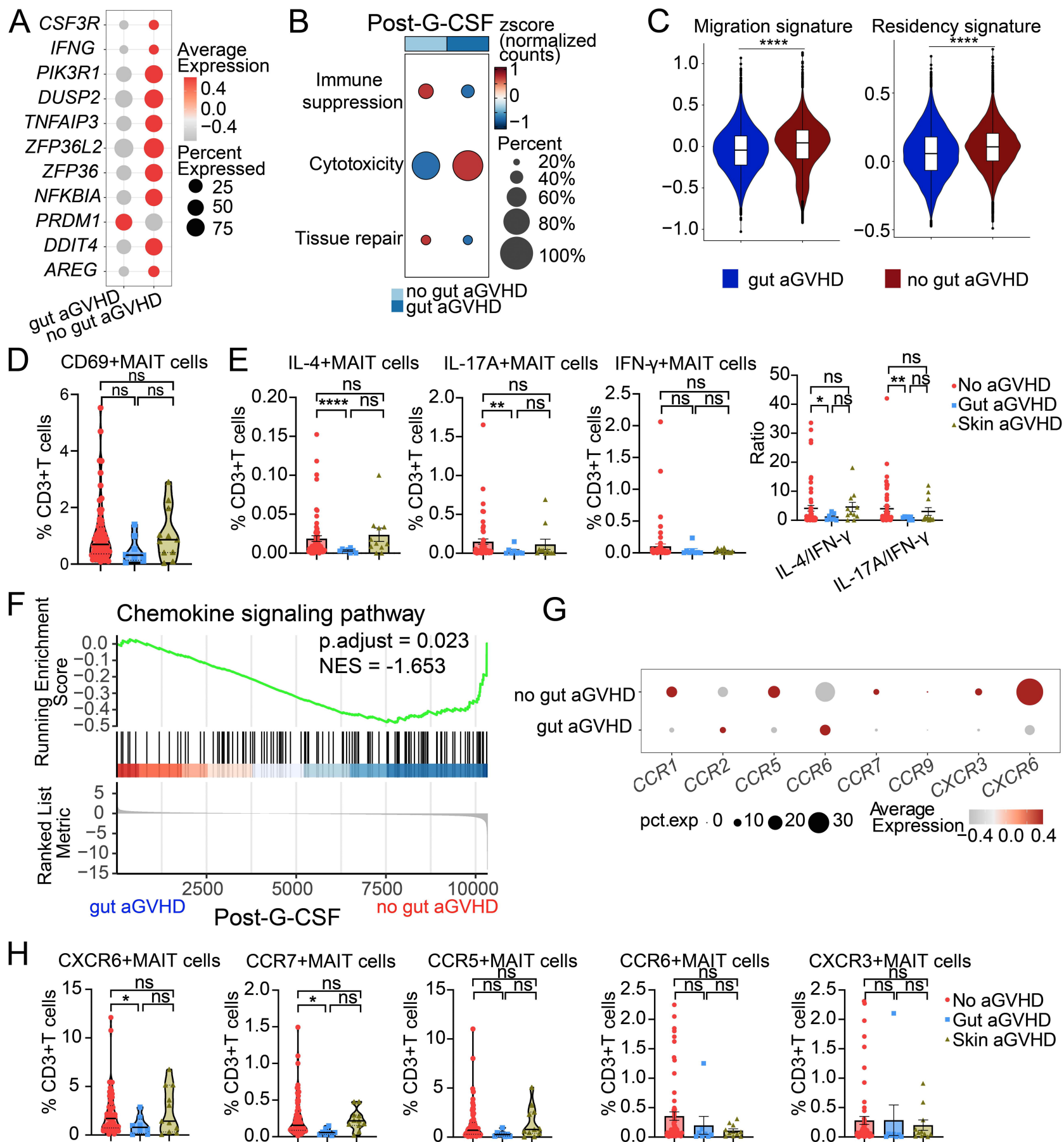
**Fig.6 Predictive panel of gut aGVHD.** **(A)** Violin box plot showing the expression scores of G-MAIT cell-related gene combination between the gut aGVHD and no gut aGVHD group (\*\*\*\* $P < 0.0001$ ). **(B)** Flow cytometry analysis showing the frequency of MAIT and  $\gamma \delta$  T cells among groups of gut aGVHD (n=9), skin aGVHD (n=13) and no aGVHD (n=55) (\* $P < 0.05$ ). **(C)** The incidence of gut aGVHD and skin aGVHD based on MAIT cell frequency. The cohort of G-PBSCs from donors (n=80) was divided into high (n=40) and low (n=40) MAIT cell frequency groups using a median cutoff of 2.95%. **(D)** MAIT cell-based scoring panel for donors after G-CSF mobilization. The panel evaluated five parameters: MAIT cell frequency (% CD3+ T cells), CD114, CXCR6, IL-17A, and IL-4. Each parameter was scored based on whether it met or exceeded the median value, with a total score ranging from 0 to 5. **(E)** The incidence of gut aGVHD and skin aGVHD in high and low score based on the flow cytometry panel in (D). A median score cutoff ( $>2$  or  $\leq 2$ ) was used to define the high (n=41) and low (n=39) score groups. **(F)** ROC curve comparison for MAIT cell proportion and the five-marker panel combination in predicting gut aGVHD. **(G)** Univariate and Multivariate analyses of factors associated with gut aGVHD occurrence.

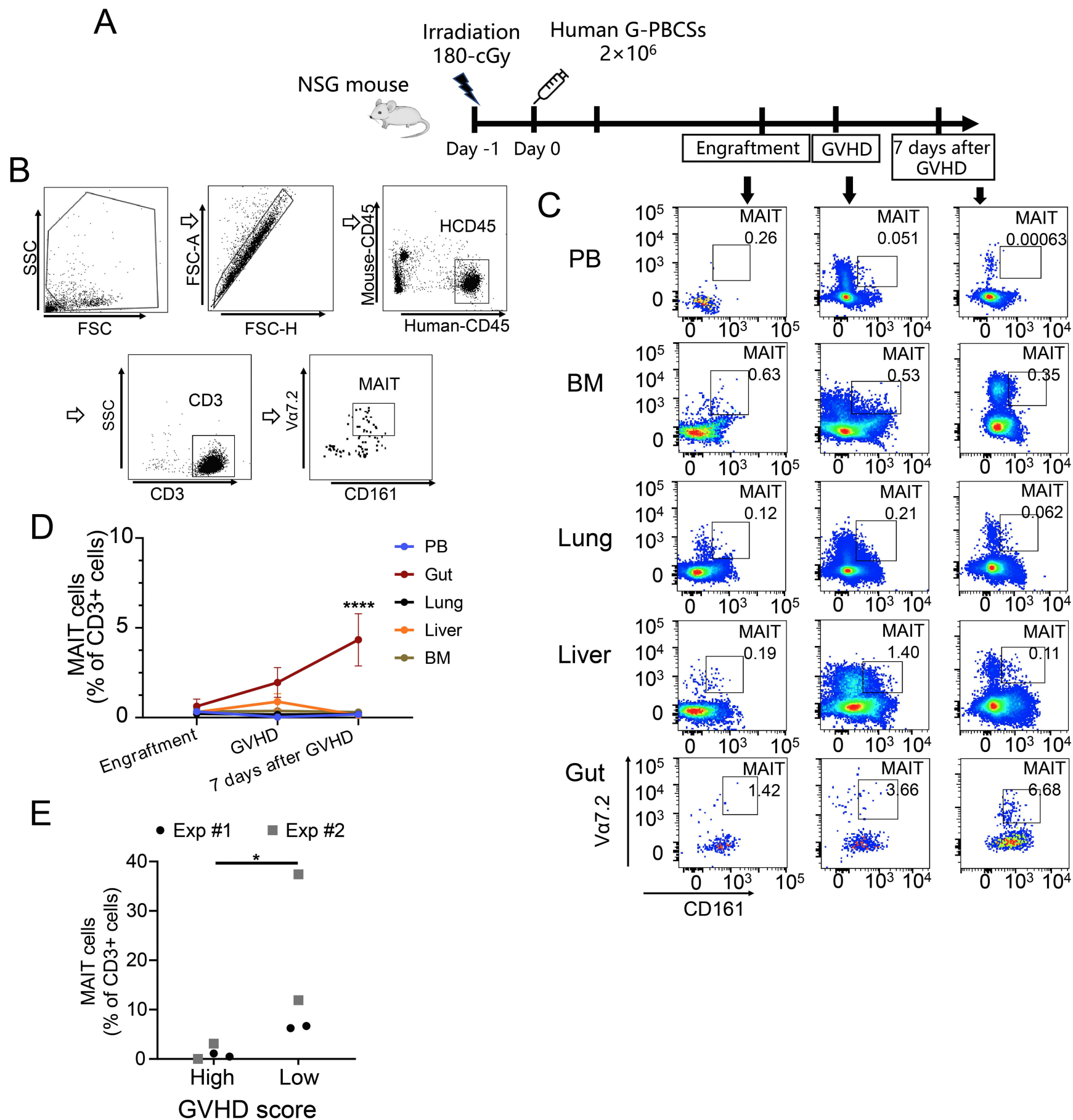
**Fig.7 The potential mechanism of anti-gut aGVHD by MAIT cells in G-PBSCs.** G-CSF binds to CSF3R on MAIT cells, stimulating them to secrete immune-modulatory and tissue-repair molecules such as IL-4 and IL-17A. Additionally, the CXCR6 receptor directs these functional MAIT cells to migrate specifically to gut pathological sites. This targeted migration enables MAIT cells to exert their immunosuppressive and tissue-repair functions, thereby countering gut aGVHD.

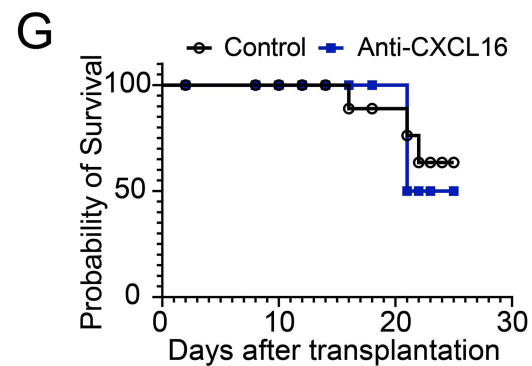
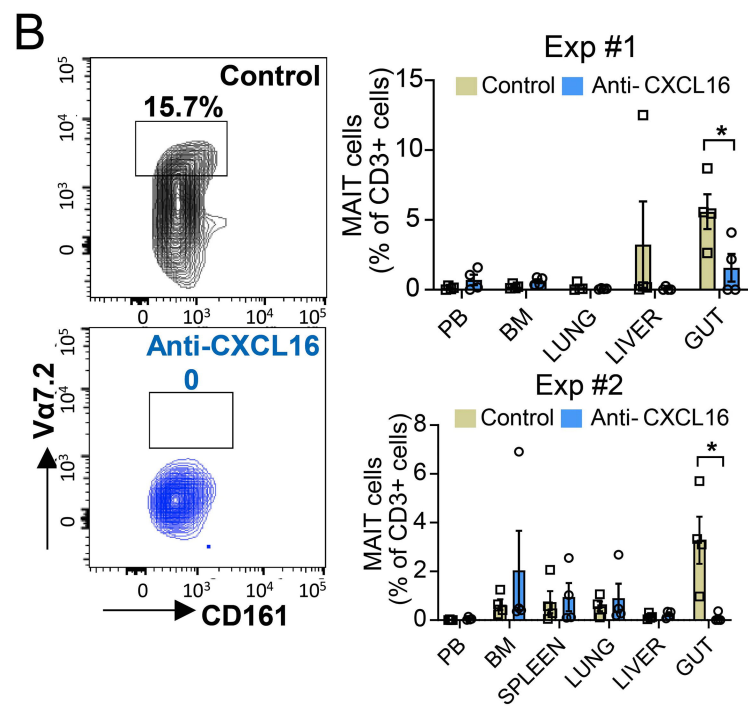
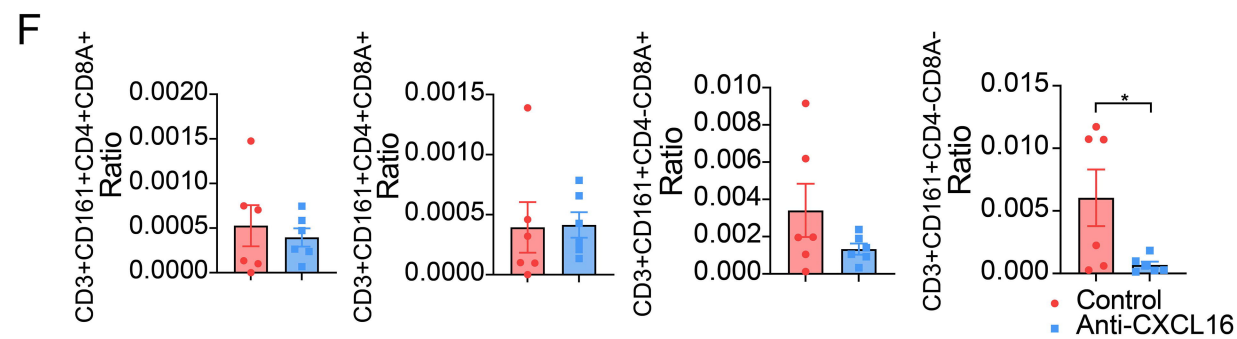
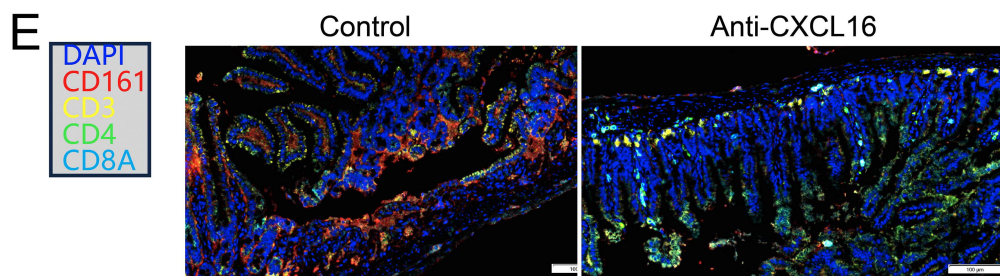
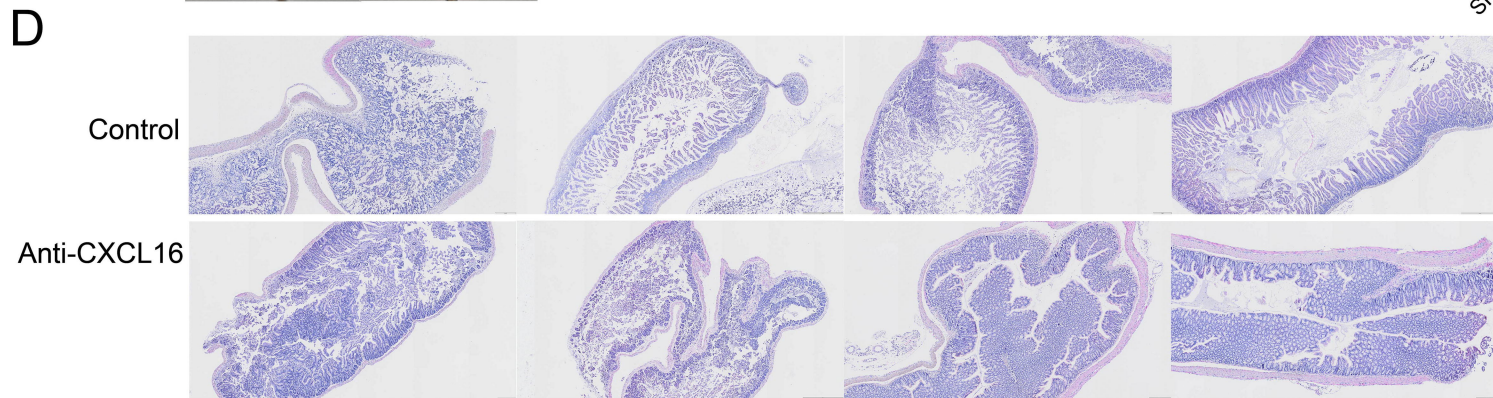
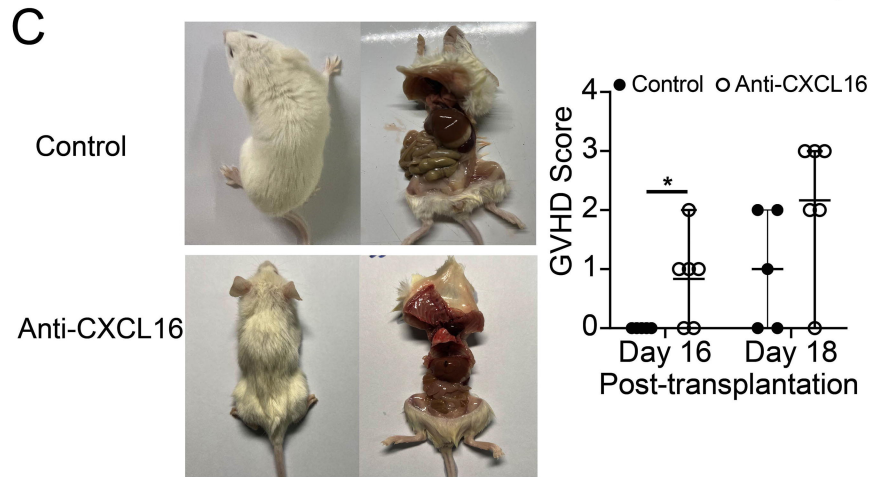
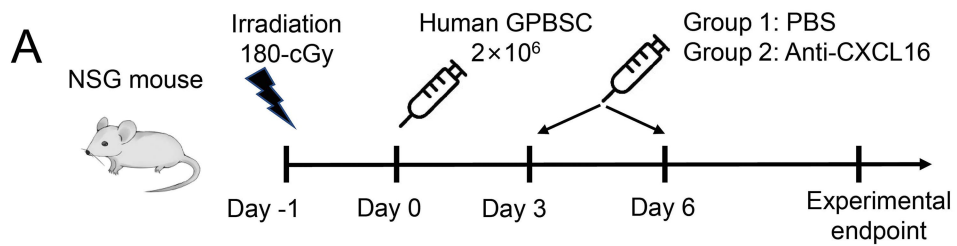
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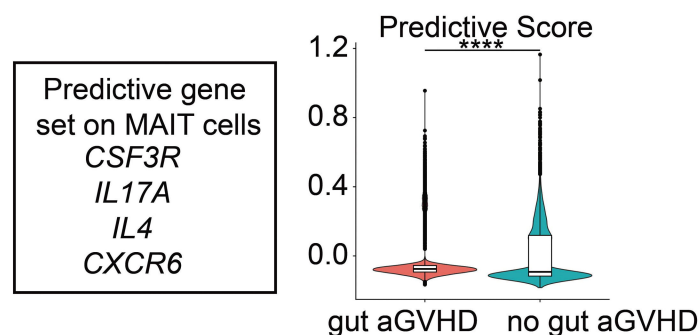




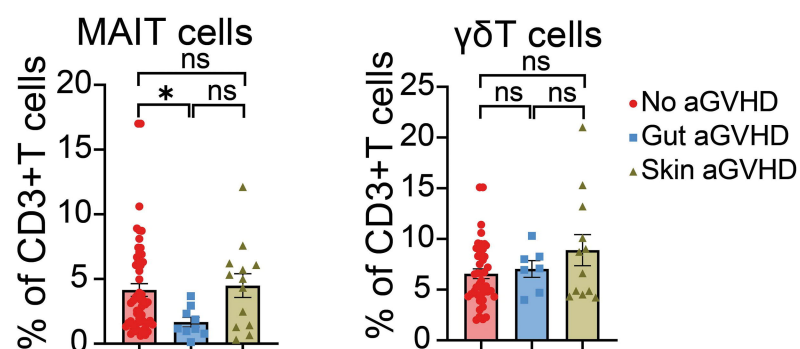




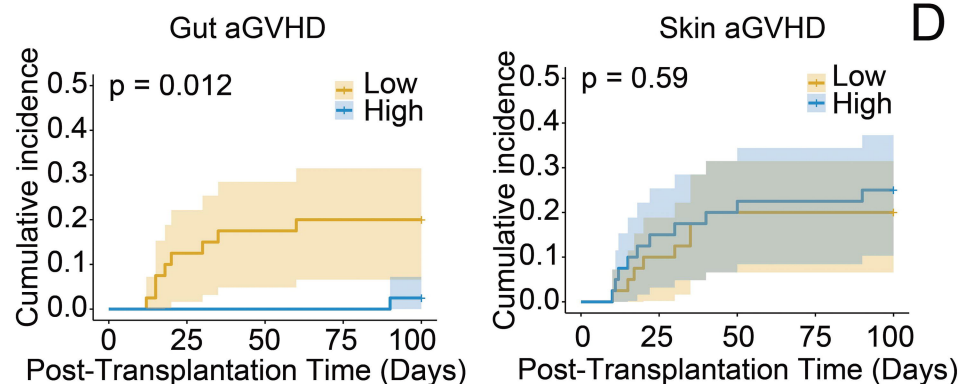
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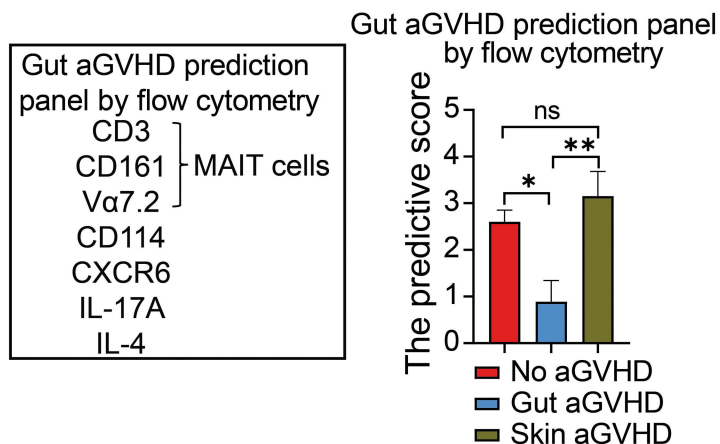
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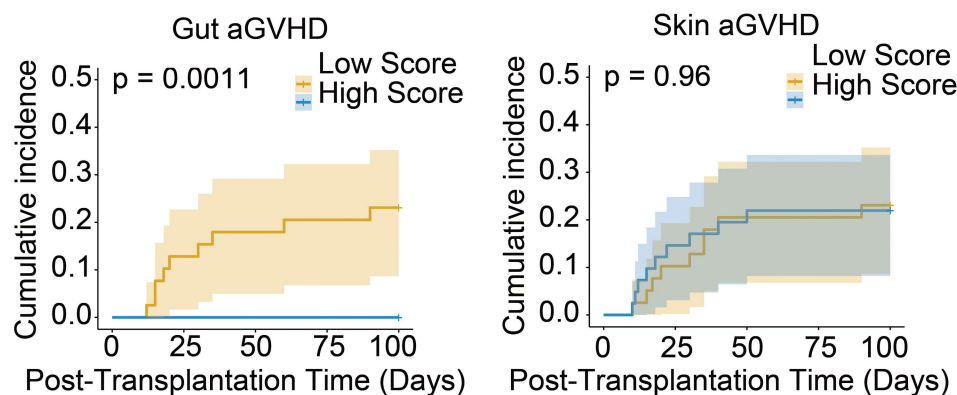
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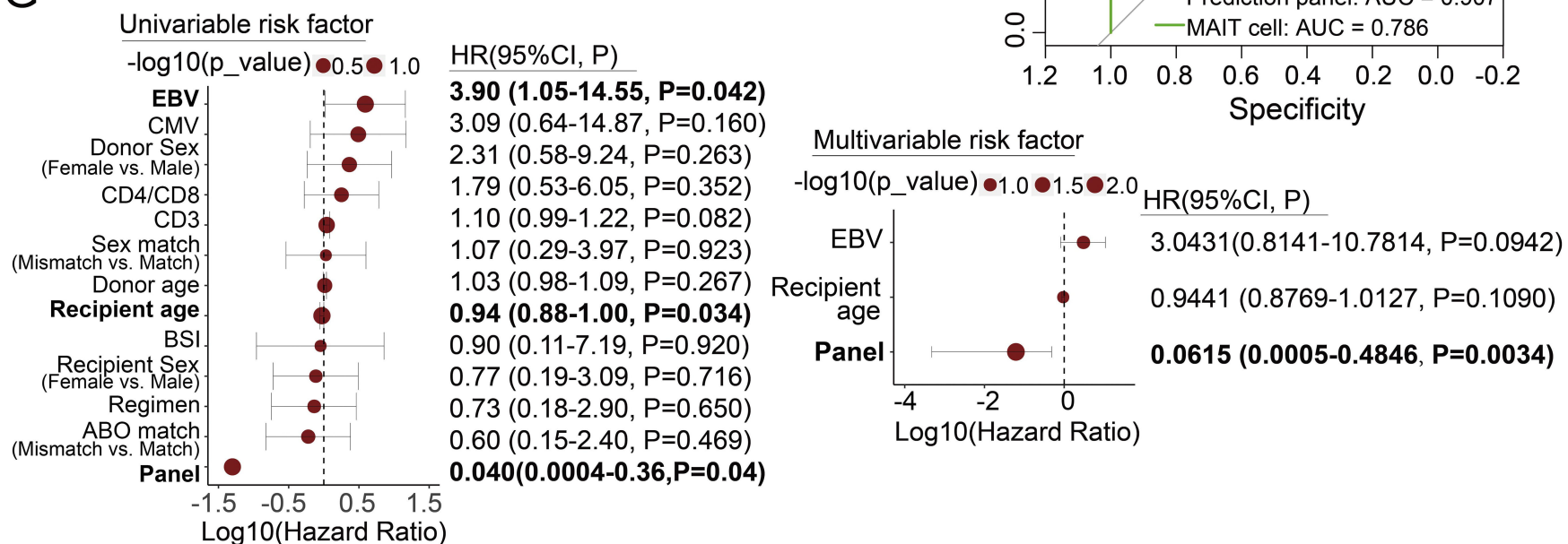
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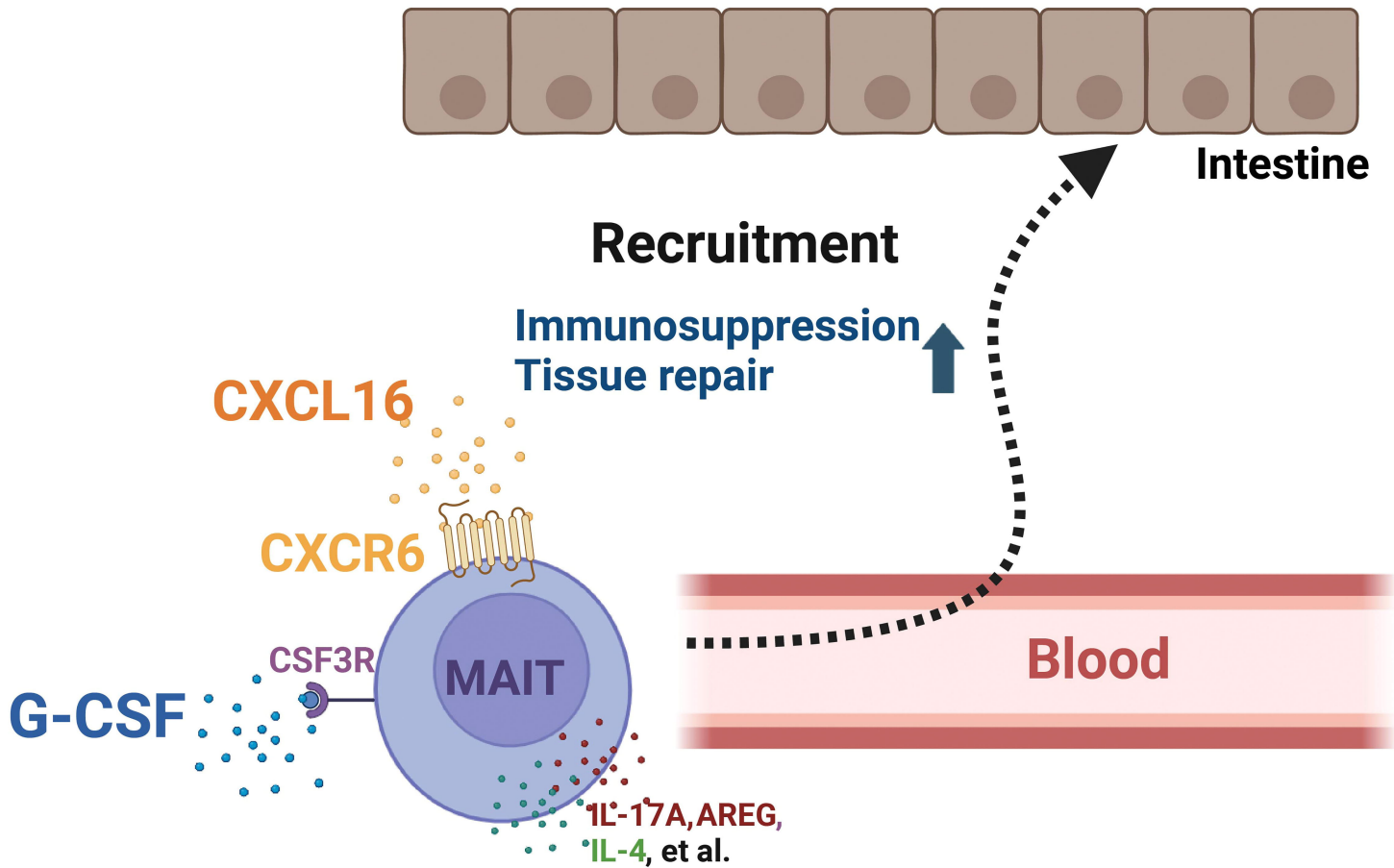


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## Supplementary Methods

### Tables 1-8 provided as Excel files only

#### Human Samples

We recruited four healthy adult donors and treated them with recombinant G-CSF (filgrastim; Kirin Co., Ltd., Tokyo, Japan) at a dosage of 5 µg/kg body weight per day for 5 consecutive days. The PBSC grafts were collected before G-CSF mobilization and after five days of G-CSF treatment, and then subjected to scRNA-seq. These donors and their matched recipients underwent haploidentical allo-HSCT at our institute between August 2020 and March 2021. Table S1 details the characteristics of donors and recipients, including sex and age. For multiparameter flow cytometry (FCM) analysis, the PB of 16 healthy donors was collected before and after G-CSF administration. In addition, a clinical cohort of 80 G-CSF-mobilized PBSC grafts (G-PBSCs) from healthy donors was also analyzed (Table S2 and Table S3).

#### Mucosa-Associated Invariant T Cell Isolation

Human PB came from healthy donors before and after *in vivo* 5-day G-CSF application, and peripheral blood mononuclear cells (PBMCs) were separated by Ficoll density centrifugation. Erythrocytes were removed by incubating with RBC lysis buffer (BD, 555899). After neutralization, the remaining cells were collected by spinning at 1500rpm for 5 min and suspended in FACS buffer (1×PBS with 1% BSA) for subsequent staining. Cells were stained in sorting buffer with MAIT cells specific antibodies for 30 min at 4 °C. MAIT cells (CD3+CD161<sup>hi</sup>Va7.2+) were isolated using fluorescence-activated cell sorting (FACS)—sorted (FACS Aria II, BD Biosciences, USA) with antibodies CD3 (Percp, Biolegend, USA), CD161 (PE-CY7, Biolegend, USA), Va7.2 (PE, Biolegend, USA) according to the manufacturer's instructions, which included one step of positive selection for CD3+ cells and next step of double positive selection for CD161<sup>hi</sup> and Va7.2+ cells.

#### Single Cell RNA Analysis

scRNA-seq libraries were prepared using the Chromium Single Cell 3' Reagent Kits v3 (10x Genomics), according to the manufacturer's instructions. The libraries were sequenced on the Illumina NovaSeq6000 platform (Novogene, provided by Berry

Genomics Corporation, Beijing, China).

For scRNA-seq data, raw gene expression matrices were generated for each sample by the Cell Ranger (version 6.0.2) pipeline coupled with human reference hg38. UMI counts were normalized by the NormalizeData function and top 2000 variable genes were calculated using the FindVariableFeatures function. Next, Principal components analysis (PCA) was performed on the scaled data, and based on the elbow plot, 30 principal components (PCs) were selected for clustering, default resolution (0.4) was used. The anchors were used for batch correction to avoid the batch effect across samples. The data were used in the subsequent nonlinear dimensional reduction with the RunUMAP function and cluster analysis by the FindNeighbors and FindClusters (dims=1:30, resolution=0.4) functions. Fifteen clusters were calculated, one of which had high expression of RBC-related genes or features, which was removed, and the remaining 14 clusters were subjected to a subsequent series of analyses.

### **Cell Type Annotation and Cluster Marker Identification**

Cells were projected into two-dimensional space using UMAP and clustered by common features. Cell clusters were annotated based on the expression of cell type-associated genes, with signature genes identified using the FindAllMarkers function in Seurat. Additionally, all percentages of different cell types were calculated.

### ***In Vitro* Proliferation and Suppression Assays**

The PBMCs from healthy adult donors were treated with or without G-CSF *in vitro*. Each  $1 \times 10^6$  PBMCs was placed in 6-well plates and cultured with 2ul G-CSF per well for 24 h, then washed with PBS and stained with antibody. CD3<sup>+</sup>CD161<sup>hi</sup>V $\alpha$ 7.2<sup>+</sup> MAIT and CD4<sup>+</sup>CD25<sup>-</sup> T cells were sorted by flow cytometry. Purified CD4<sup>+</sup> T lymphocytes were labeled with  $5 \times 10^{-6}$  m 5,6-carboxy-fluorescein diacetate succinimidyl ester (CFSE) (BD Bioscience) for 15 min at 37 °C and washed twice with complete medium with 10% FBS (fetal bovine serum). Isolated G-CSF mobilized and non-mobilized MAIT cells were co-cultured with labeled CD4<sup>+</sup>T cells ( $2 \times 10^4$  cells per well) at MAIT/CD4<sup>+</sup>T ratios of 1:1, 2:1, and 4:1 in the presence of Dynabeads Human T-Activator CD3/CD28 beads (Thermo, 11132D) in RPMI

complete medium (Gibco, Invitrogen) in flat-bottomed 96-well plates. After 96 h, cells were harvested for flow cytometry analysis.

### **The Killing Assays of MAIT Cells On Tumor Cell Lines**

Isolated MAIT cells by flow cytometry were treated with or without G-CSF for 24 h. Then, G-CSF mobilized and non-mobilized MAIT cells were co-cultured with K562 tumor cell lines at MAIT/K562 ratios of with 1:1. MAIT cells ( $2 \times 10^5$  cells per well) were stimulated with cytokines (IL-12/IL-18, PeproTech) and/or anti-CD3/CD28 beads (Thermo) in 96-well U plate in RPMI 1640 (Gibco, Invitrogen) added 10% FBS. After 96 h, tumor cell apoptosis was assessed by flow cytometry using 7-AAD and Annexin V staining.

### **GVHD Mouse Models**

NOD-SCID-IL2R $\gamma^{-/-}$  (NSG) mice were used to establish the xenogeneic GVHD model. All animals were bred and maintained under specific pathogen-free conditions in microisolator cages and used at 6–8 weeks of age in accordance with protocols approved by the local Ethics Committee. On day -1, mice received sublethal total body irradiation (180 cGy, X-ray), followed by intravenous injection of  $2 \times 10^6$  human G-PBSCs via the tail vein. Human MAIT cells were assessed in peripheral blood, gut, liver, lung, and bone marrow at engraftment, GVHD onset, and day 7 post-GVHD. Mice were monitored two to three times weekly for survival, body weight, and GVHD clinical score. GVHD scoring was based on six parameters—skin integrity, weight loss, posture, activity, fur texture, and diarrhea—each graded 0 or 1, with a maximum score of 6.

### **CXCL16 Blocking in NSG Mice**

*In vivo* CXCL16 blocking was done by injecting i.e. 100  $\mu$ g of anti-CXCL16 antibody (R&D, AF976) or PBS twice within a week after transplantation. Mice were monitored for survival, weight, and acute GVHD score was evaluated two or three times a week. The endpoint was defined as: (1) natural death; (2) mice showing 20% weight loss and clinical symptoms of GVHD; (3) 24 days after transplantation.

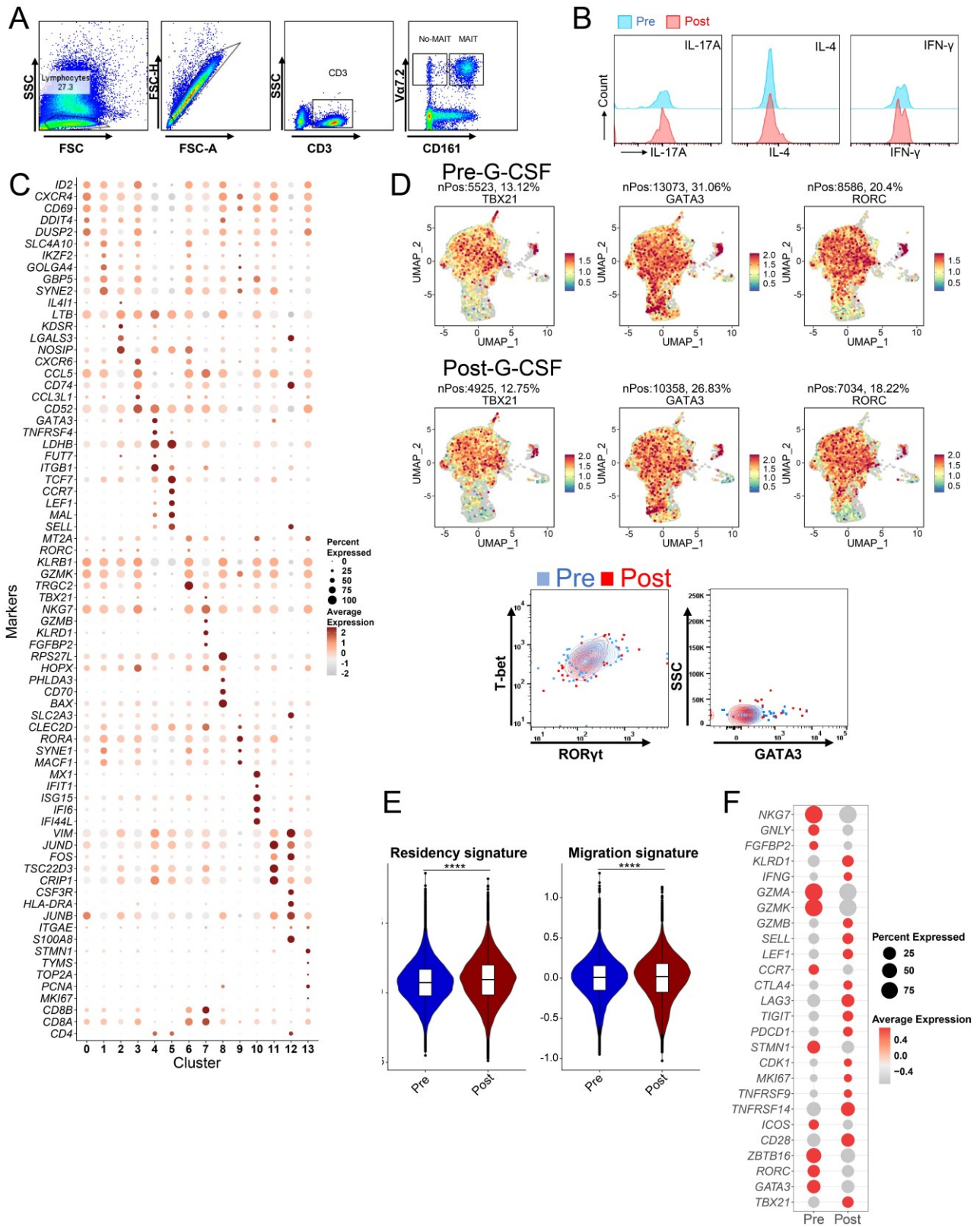
### **Immunofluorescence**

Intestinal tissues from aGVHD NSG mice were processed for immunofluorescence analysis. Paraffin-embedded samples were sectioned into 3–4  $\mu\text{m}$  slices and mounted on polylysine-coated slides. Paraffin sections were baked overnight at 58°C, dewaxed in xylene, rehydrated through a graded series of ethanol, quenched for endogenous peroxidase activity in 0.3% hydrogen peroxide for 15 mins. Antigen retrieval was performed by high-pressure cooking in citrate buffer (pH=6.0) for about 20 mins, then allowed to cool to room temperature, blocking the nonspecific antibody binding sites in 5% normal goat serum for 2 hrs. Primary antibodies, including CD3 (1:100, Abcam, ab135372, UK), CD4 (1:50, ZSGB-BIO, ZM-0418, China), CD8 (1:100, CST, CST70306, USA), and CD161 (1:100, Abcam, ab197979, UK), were applied at room temperature for 1 hour. The EnVision+ System-HRP (AEC) (K4005, Dako, Denmark) was used to detect the primary antibodies, followed by hematoxylin counterstaining (Sigma-Aldrich, USA). TMA slides were scanned using an automated microscope, and quantitative analysis was performed with Image-Pro Plus software (IPP, Media Cybernetics, USA).

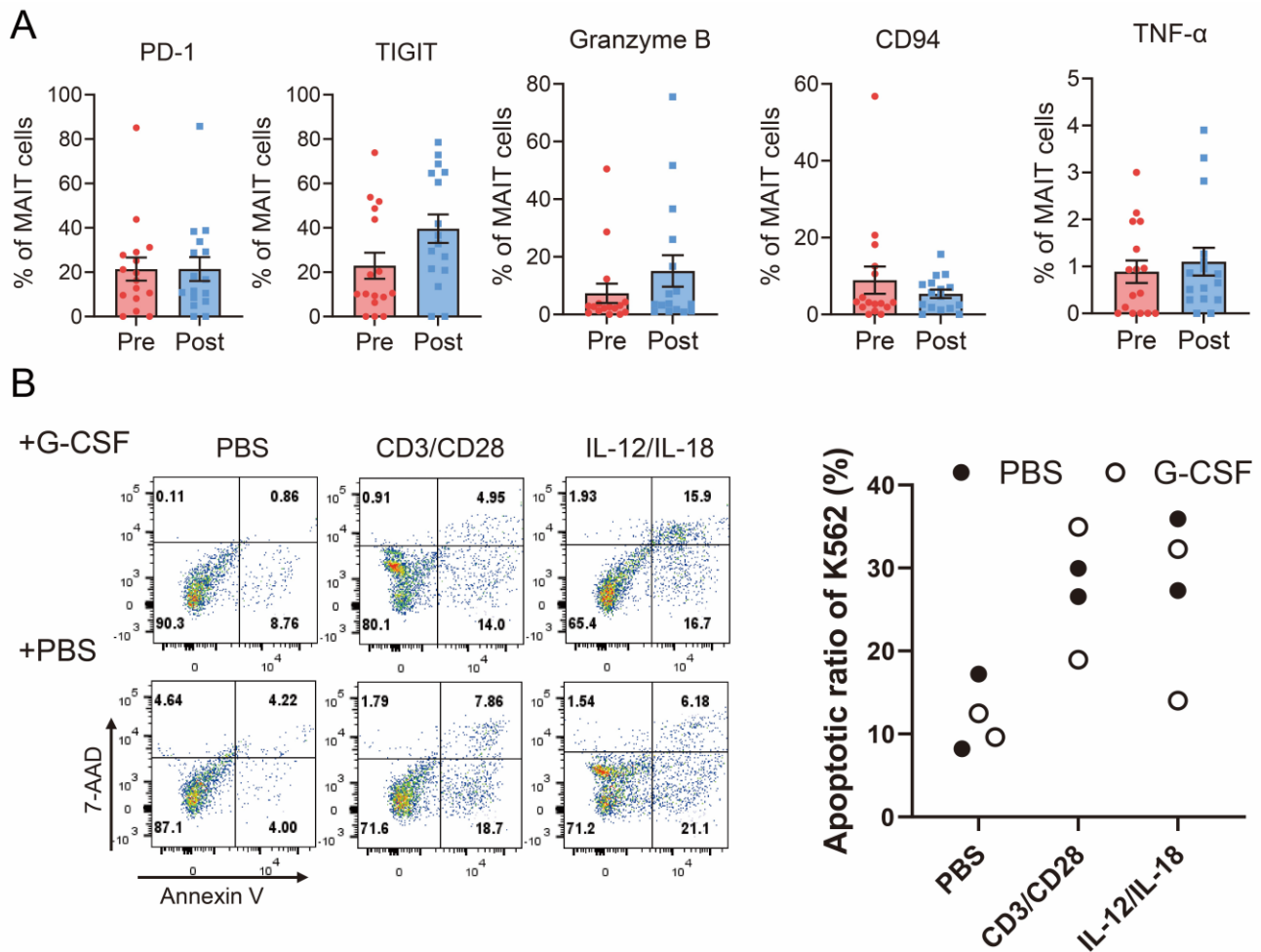
### **Calculation of predictive scores**

The frequencies of MAIT cells and their functional subsets (CD114+, IL-17A+, IL-4+, and CXCR6+ MAIT cells) were quantified by multicolor flow cytometry, with all samples processed using consistent staining, acquisition, and analysis protocols. To determine robust thresholds, we applied bootstrap resampling (1000 iterations) to the clinical cohort, calculating the median proportion of each subset per iteration; the mean of these medians was defined as the bootstrap median threshold. For each patient, a binary score was assigned per subset: 1 point if the proportion was equal to or above the bootstrap median, and 0 if below. The sum of these five binary scores yielded a composite score ranging from 0 to 5.

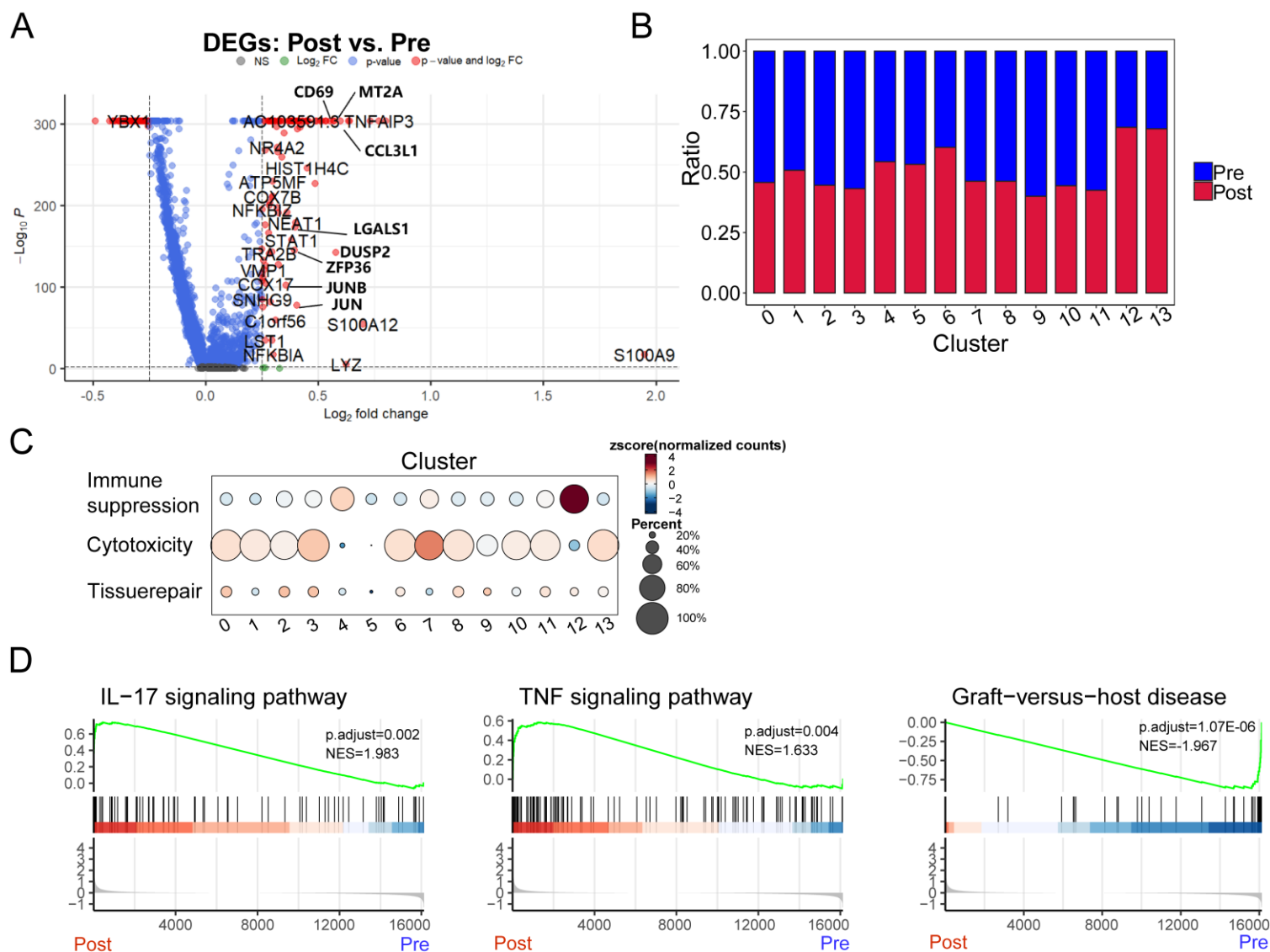
## Supplementary Figures



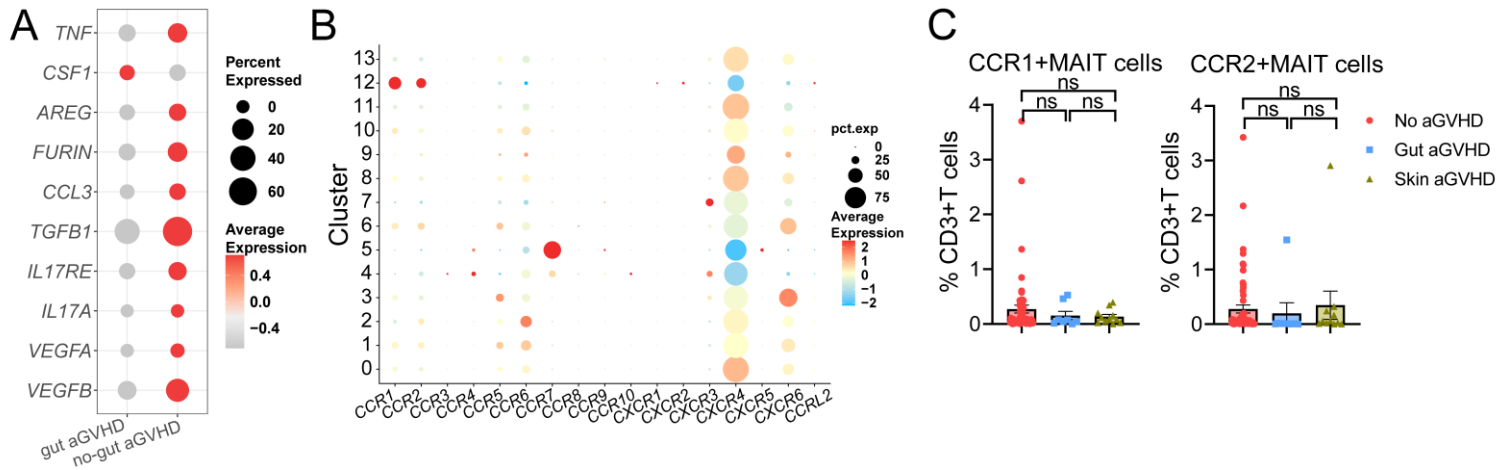
**Supplementary Figure 1. Effect of G-CSF on functional molecule expression in MAIT cells.** (A) Gate strategy of human MAIT cell. (B) Representative flow cytometry showing the expression levels of IL-4 ( $P=0.041$ ), IFN- $\gamma$  and IL-17A in MAIT cells from donor PBSCs (n=16) before and after G-CSF-mobilization. (C) Dot plot displaying key marker genes for each MAIT cell cluster among the differentially expressed genes (DEGs). Dot size and color intensity represent the proportion of expressing cells and expression levels, respectively. (D) UMAP plot and Flow cytometry showing the expression of transcription factors T-bet, GATA3 and ROR $\gamma$ t for each cell. (E) Violin Box plot showing migration and residency signatures in MAIT cells before and after G-CSF-mobilization (\*\*\*\* $P$  value < 0.0001). (F) Dot plot showing the expression of functional molecules in MAIT cells before and after G-CSF mobilization.



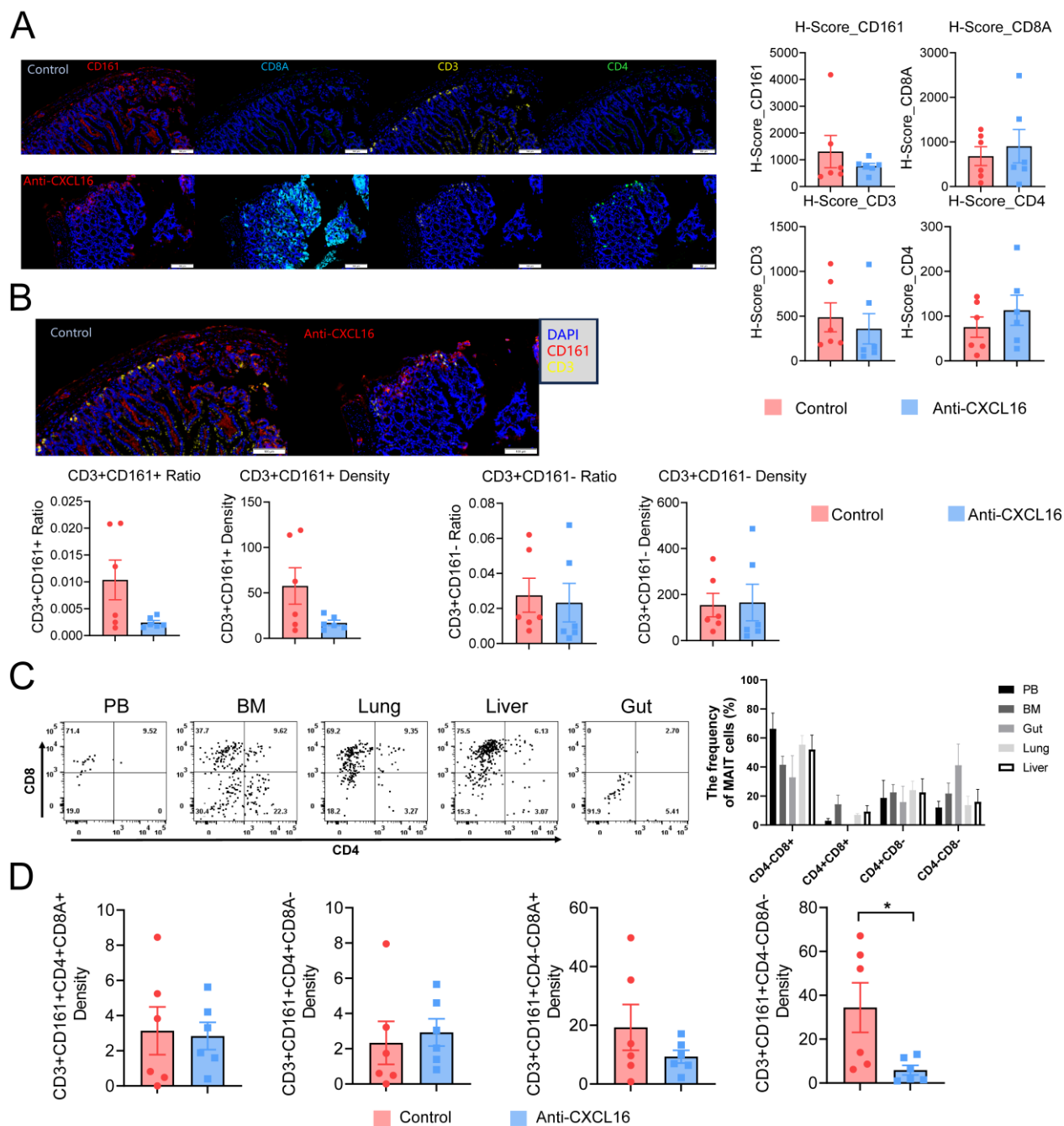
**Supplementary Figure 2. (A)** Flow cytometric analysis of expression of functional molecules in MAIT cells from donor PB (n=16) before and after G-CSF-mobilization. **(B)** Representative flow cytometry results showing the effect of G-CSF mobilization on the cytotoxic activity of MAIT cells against K562 cell line, following stimulation by cytokine (IL-12/IL-18) and TCR (CD3/CD28) pathway.



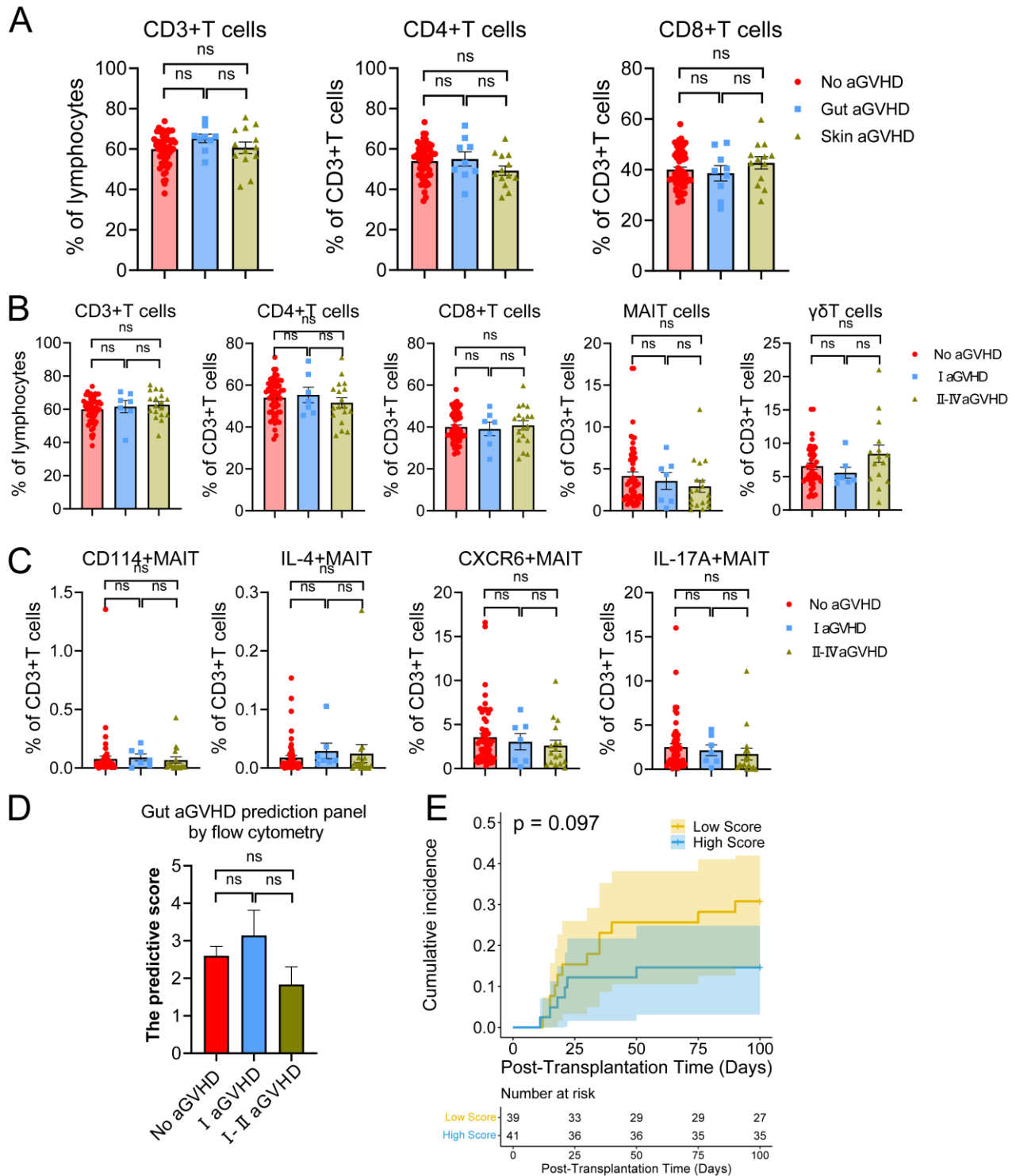
**Supplementary Figure 3. The effect of G-CSF on MAIT cells within Cluster 12 expressing CSF3R. (A)** EnhancedVolcano showing the DEGs of MAIT cells between unstimulated MAIT and G-CSF mobilized MAIT cells. DEGs were detected by differential expression analysis (two-sided Student's t-test). Each dot represents a single gene. **(B)** The proportion of MAIT cell clusters before and after G-CSF mobilization. **(C)** Dot plot showing functional signatures distribution in MAIT cells clusters. **(D)** Gene set enrichment analysis (GSEA) showing the major enriched terms of DEGs before and after G-CSF administration.



**Supplementary Figure 4. The characteristics of tissue repair and chemotactic migration of MAIT cells may be involved in its anti-intestinal GVHD effect. (A)** Dot plot showing the average expression of cell tissue repair-related genes on G-MAITs between groups of gut aGVHD (n=1) and no gut aGVHD (n=3). **(B)** Dot plot showing the expression of chemokine receptor genes on MAIT clusters. **(C)** Flow cytometric analysis showing chemokine receptor expression in G-MAIT cells between groups of gut aGVHD (n=9), skin aGVHD (n=13) and no aGVHD (n=55).



**Supplementary Figure 5. Distribution of MAIT cells in G-grafts within NSG mouse transplantation models. (A)** Single-marker immunofluorescence and histological score (H-score). **(B)** Immunofluorescence showing the ratios and densities of CD3+CD161+ and CD3+CD161- cell populations in the intestinal tissues of control and anti-CXCL16 groups. Density = Number of positive cells / Tissue area ( $\text{mm}^2$ ). **(C)** Flow cytometric analysis showing CD4 and CD8 expression of MAIT cells in PB, BM and recipient tissues post-transplantation. **(D)** Immunofluorescence showing population densities in the intestinal tissues of control and anti-CXCL16 groups.



**Supplementary Figure 6. The relationship between predictive signatures in MAIT cells and aGVHD severity.** (A) Flow cytometry analysis showing the frequency of T cells and subsets among groups of gut aGVHD (n=9), skin aGVHD (n=13) and no aGVHD (n=55). (B) Flow cytometry analysis showing the frequency of T cells and subsets among groups stratified by aGVHD grade (I aGVHD: n=7; II-IV aGVHD: n=18). (C) GVHD-related functional gene of MAIT cells were compared between patients stratified by aGVHD grade. (D) The GVHD scores were compared between patients stratified by aGVHD grade. (E) Kaplan-Meier analysis showing

the incidence of II–IV aGVHD in high and low score from predictive scores.