



Characteristics of peripheral blood mononuclear cells and potential related molecular mechanisms in patients with autoimmune hepatitis: a single-cell RNA sequencing analysis

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Received: 17 November 2023 / Accepted: 3 January 2024 / Published online: 10 February 2024
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Abstract

Autoimmune hepatitis (AIH) is an immune disorder characterized by hypergammaglobulinemia, autoantibodies, and chronic active hepatitis on liver histology. However, immune cell population characteristics in AIH patients remain poorly understood. This study was designed to analyze peripheral blood mononuclear cell (PBMC) characteristics in AIH through single-cell RNA sequencing (scRNA-seq) and explore potential AIH-related molecular mechanisms. We generated 3690 and 3511 single-cell transcriptomes of PBMCs pooled from 4 healthy controls (HCs) and 4 AIH patients, respectively, by scRNA-seq. These pooled PBMC transcriptomes were used for cell cluster identification and differentially expressed gene (DEG) identification. GO functional enrichment analysis was performed on the DEGs to determine the most active AIH immune cell biological functions. Although the PCA-based uniform manifold approximation and projection (UMAP) algorithm was used to cluster cells with similar expression patterns in the two samples, 87 up- and 12 downregulated DEGs were retained in monocytes and 101 up- and 15 downregulated DEGs were retained in NK cells from AIH PBMCs. Moreover, enriched GO terms in the PBMC-derived monocyte and NK cell clusters were related mainly to antigen processing and presentation, IFN- γ -mediated signaling, and neutrophil degranulation and activation. These potential molecular mechanisms may be important targets for AIH treatment.

Keywords Autoimmune hepatitis · Single-cell RNA sequencing · PBMCs · Natural killer cells · Monocytes

Introduction

Autoimmune hepatitis (AIH) can present as chronic hepatitis of unknown cause. AIH is commonly associated with the presence of autoantibodies and hypergammaglobulinemia. Histologically, interface hepatitis with marked lymphocyte and plasma cell infiltration was observed. AIH is a disease that develops and progresses due to autoimmune reactions caused by a failure in immunological tolerance to hepatocytes, and it is speculated that various genetic and immunological abnormalities are involved [1–5].

Although the etiology of AIH is unclear, it is supposed that environmental and genetic factors are involved in its pathogenesis. The role of gene–environment interactions has also been predicted in autoimmune diseases. Viral infections, low levels of vitamin D, altered microbiota, exposure to sex hormones, and the administration of drugs are candidate environmental factors that can predispose an individual to AIH [6–8]. The nature of AIH is complex and may depend on interactions between genetic susceptibility and environmental triggers [9]. A genome-wide association study (GWAS) is an association study of a genome-wide set of single-nucleotide variants (SNVs) to determine the contribution of common variants to susceptibility to disease. A GWAS of type 1 AIH was performed in European populations, and a significant association of SNVs in the human leukocyte antigen (HLA) region was found, indicating that HLA-related factors are the strongest genetic risk factors for AIH [10].

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Immunity-related genes play essential roles in the immune response; however, the expression of immunity-related genes and possible the regulatory mechanisms involved in AIH remain unclear. *HLA*, *STAT4*, *TNIP1*, *TNFAIP3*, *SH2B3*, and *PTPN22* are common susceptibility genes for AIH in the Japanese population [11]. According to HLA association studies, *DRB1*04:01* and *DRB1*04:05* are associated with AIH in the Japanese population [12–15]. Lymphoid-specific protein tyrosine phosphatase is encoded by *PTPN22* and plays an important role in the regulation of T cell activation. *SH2B3* is an adaptor protein regulating cytokine signaling and a negative regulator of T cell activation [16].

Advances in next-generation sequencing technologies have recently made it possible to study the immune system at the single-cell level. In 2009, Tang et al. [17] first published single-cell transcriptome sequencing (i.e., scRNA-seq) technology, which has enabled high-resolution mapping of cellular heterogeneity, development, and activation states in diverse systems. This technique is highly important for the discovery of new therapeutic targets for autoimmune diseases. Currently, there is only one report on the use of scRNA-seq analysis of peripheral blood mononuclear cells (PBMCs) in AIH; specifically, studies on autoreactive CD4+ T cells following interaction with B lymphocytes [18].

The present study was designed to analyze the characteristics of PBMCs from patients with AIH through scRNA-seq and to explore potential AIH-related molecular mechanisms.

Materials and methods

Study population and sample collection

First, we selected four patients with AIH and four healthy controls (HCs) to identify differences in PBMC profiles between these groups through the use of scRNA-seq. The participants were four patients with AIH diagnosed at Fukushima Medical University Hospital between 1993 and 2020. Normal serum was collected from staff members at our hospital as HCs. The diagnosis of AIH was based on the revised and simplified International Autoimmune Hepatitis Group (IAIHG) scoring system [19–21]. Autoantibodies (antinuclear antibodies [ANAs] and anti-smooth muscle antibodies [anti-SMAs/ASMAs]) were detected by indirect immunofluorescence in HEp-2 cells or frozen sections of rat kidney tissue. The anti-liver/kidney microsomal 1 (LKM-1) antibody concentration in the serum was measured via enzyme-linked immunosorbent assay (ELISA) using commercially available kits. Patients with chronic liver disease due to other causes, particularly alcohol abuse, chronic hepatitis B virus (HBV) infection, or HCV infection, were excluded from the AIH group. Patients with AIH were excluded if they

tested positive for Epstein–Barr virus (EBV), cytomegalovirus (CMV), hepatitis A virus (HAV), or hepatitis E virus (HEV) infection as assessed by the presence of EBV IgM, CMV IgM, HAV IgM, or HEV IgA, respectively. Those were retrospectively extracted from the patients' medical charts included background characteristics (age, sex); clinical parameters at presentation (alanine aminotransferase [ALT], alkaline phosphatase [ALP], total bilirubin [TB], and albumin [ALB] levels; platelet [PLT] count; prothrombin time [PT]; and IgG and ANA); the presence of relapse; and therapeutic methods. AIH relapse was defined as an increase in the serum transaminase concentration to more than twice the upper limit of normal (ALT level > 90 U/L). Biochemical remission was defined as complete normalization of aminotransferase levels and IgG titers [22, 23].

All the samples were thawed on the same day and immediately processed with 10× Genomics Gene Expression 3' Chromium V 2.0 per the manufacturer's instructions. We generated single-cell transcriptomes of PBMCs that were pooled from four HCs and four AIH patients by scRNA-seq.

Histological evaluation

Four patients with AIH underwent ultrasound-guided liver biopsy. Liver sections were stained with hematoxylin and eosin (H&E). Slides were coded and read by two pathologists blinded to patient identity and history. Histologic evaluation was performed according to the classification of Scheuer [24] and Desmet et al. [25]. The grades of necro-inflammatory activity (G) and stages of fibrosis (S) ranged from G0 to G4 and from S0 to S4, respectively.

Library preparation and sequencing

Peripheral blood was drawn from each patient and volunteer into a tube containing EDTA-2Na, centrifuged to separate the PBMCs and then frozen. A single-cell suspension was obtained by filtering the cell suspension with a 40 µm cell strainer. The isolated single cells were loaded onto a 10× Chromium Controller (10× Genomics). Single-cell cDNA synthesis and amplification were performed, and sequencing of the libraries was performed using the Single-cell 3' Library Kit v3.1 following the manufacturer's protocol. The libraries were sequenced on a NovaSeq 6000 (Illumina) platform with the following sequencing parameters: 28 bp read 1, 8 bp index 1, and 91 bp read 2.

Sequence data processing

Sequenced reads were subjected to de-multiplexing, alignment, barcode counting, unique molecular identifier (UMI) counting, and filtering using Cell Ranger v.5.0.0. The human

genome (GRCh38) was used as a reference for aligning reads.

Cell clustering

Dimensionality reduction was performed using principal component analysis (PCA). The first 50 principal components were used to cluster the cells based on the shared nearest neighbors algorithm (SNN) and visualized in two dimensions by the uniform manifold approximation and projection (UMAP) algorithm [26]. For each cluster, the marker genes were identified using the FindMarkers function as implemented in the Seurat package ($\log_{fc}.\text{threshold} > 0.25$ and $\text{minPct} > 0.2$). Clusters were subsequently assigned to known cell types [27].

Differential gene expression and functional enrichment

Differentially expressed genes (DEGs) across different samples or clusters were identified as those with \log fold changes > 0.25 and adjusted p values < 0.001 . Gene Ontology (GO) analysis was performed using gene set enrichment analysis (GSEA) with a false discovery rate (FDR) ≤ 0.05 considered to indicate significant enrichment. Gene expression analysis was performed by Genble, Inc., Fukuoka, Japan.

Statistical analysis

The following analyses were performed in R (version 3.6.0) using the package Seurat (version 3.1.4). Genes expressed in fewer than three cells were removed. Cells that expressed fewer than 200 genes or more than 5000 genes were removed. We also removed the cells that had more than 15% of the genes associated with mitochondria among their

expressed genes and had more than 30,000 UMIs. The UMIs were \log -transformed (scale factor = 10,000). We regressed out the S scores, G2/M scores, and the percentage of mitochondrial-associated genes during data scaling. Dimensionality reduction was performed using principal component analysis (PCA). The first 50 principal components were used to cluster the cells based on the shared nearest neighbors (SNN) algorithm and visualized in two dimensions by uniform manifold approximation and projection (UMAP). Differential gene expression analysis was performed using MAST in the Seurat package with a min.pct value of 0.2. DEGs between different samples or clusters were identified as those with a \log fold change > 0.25 and an adjusted p value < 0.001 . GO analysis was performed using GSEA, with an FDR of ≤ 0.05 indicating significant enrichment.

Data availability

The datasets generated and analyzed during the current study are available in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) repository [Accession Number: GSE216064].

Results

Patient characteristics

The baseline characteristics of the patients with AIH are summarized in Table 1. Four patients with AIH tested positive for ANAs. All patients experienced relapse during immunosuppressive therapy. The distribution of necro-inflammatory activity grades was as follows: G0, 0/4; G1, 0/4; G2, 4/4; 100%; G3, 0/4; and G4, 0/4. The distribution of fibrosis stages was as follows: S0 (0/4, 0%), S1 (3/4, 75%), S2 (0/4, 0%), S3 (1/4, 25%), and S4 (0/4, 0%). Regarding

Table 1 Characteristics of AIH patients and HCs

	AIH at diagnosis	AIH at remission	HC
Age (year), median (IQR)	47.5 ± 15.3	54.2 ± 8.6	46.0 ± 2.9
Sex male/female	0/4	0/4	0/4
ALB, g/dL, median (IQR)	3.8 ± 0.3	4.1 ± 0.1	NA
TB, mg/dL, median (IQR)	2.5 ± 2.6	0.6 ± 0.1	NA
PT, % (IQR)	64.2 ± 29.2	106.5 ± 15.0	NA
ALT, U/L, median (IQR)	377.2 ± 282.2	34.2 ± 39.8	NA
ALP, U/L, median (IQR)	461.7 ± 103.4	235.5 ± 65.7	NA
IgG, mg/dL median (IQR)	2533 ± 837	1289 ± 256	NA
Staging of fibrosis 0/1/2/3/4	0/3/0/1/0		NA
Grading of activity 0/1/2/3/4	0/0/4/0/0		NA
Relapse, n (%)		4 (100)	NA
PSL/AZA combination therapy, n (%)		4 (100)	NA

NA not applicable

therapy, all patients were treated with azathioprine in combination with prednisolone (PSL).

scRNA profiling of PBMCs from AIH patients

After stringent quality control and filtering by multiple criteria, transcriptomes of 3,511 and 3,690 single cells from the AIH patient and HC samples, respectively, were acquired, with a mean of 1970 and 1768 genes per cell detected, respectively (Supplemental Table S1). The UMAP algorithm was subsequently used to cluster cells with similar expression patterns via PCA. In this study, the two samples were classified into 16 clusters (Fig. 1a). According to a previous study, these clusters could be assigned to known cell lineages through marker genes [28–30]. The expression of cell type marker genes is shown in the violin plot (Fig. 1b). For the two samples, the 16 cell clusters were as follows: naïve CD4 + T cell 1, CD4 + T cell 1, monocyte, natural killer (NK) cell 1, effector CD8 + T cell, memory B cell, naïve CD8 + T cell, memory CD8 + T cell 1, memory CD8 + T cell 2, CD4 + T cell 2, naïve B cell, naïve CD4 + T cell 2, monocyte (nonclassical), dendritic cell, NK cell 2 and platelet (Fig. 1c, d).

The number and proportion of cells in each cluster are shown in Fig. 1e. We observed that the proportions of the main cell clusters shifted in AIH patients, with increased proportions of monocytes, NK cells, and effector CD8 + T cells and a decreased proportion of naïve CD4 + T cells (Supplemental Table S2).

Patients with AIH exhibit upregulated gene expression in monocytes and NK cells

To investigate the expression patterns of PBMCs in AIH, the scRNA-seq dataset (GSE216064) from the GEO database, which included data from 4 AIH patients and 4 HCs, was analyzed to explore DEGs in PBMCs from AIH patients and HCs. DEGs with adjusted P values < 0.001 and average logFC > 0.25 were selected. A heatmap of the top 120 upregulated and downregulated DEGs is shown, and relative consistency was observed within groups (Fig. 2a). We defined genes with upregulated or downregulated expression in AIH patients compared with HCs as DEGs (Fig. 2b). By analyzing the expressed genes, we found that two DEGs [FK506-binding protein 5 (*FKBP5*) and ribosomal protein S26 (*RPS26*)] were shared by naïve CD4 + T cells, CD4 + T cells, monocytes, NK cells, effector CD8 + T cells, memory B cells, naïve CD8 + T cells, and memory CD8 + T cells; two DEGs [thioredoxin-interacting protein (*TXNIP*) and nuclear enriched abundant transcript 1 (*NEAT1*)] were common to naïve CD4 + T cells, CD4 + T cells, naïve CD8 + T cells, and memory CD8 + T cells; and a single DEG (*HLA-DRB5*) was

common to monocytes, NK cells, effector CD8 + T cells, and memory B cells (Supplemental Table S3).

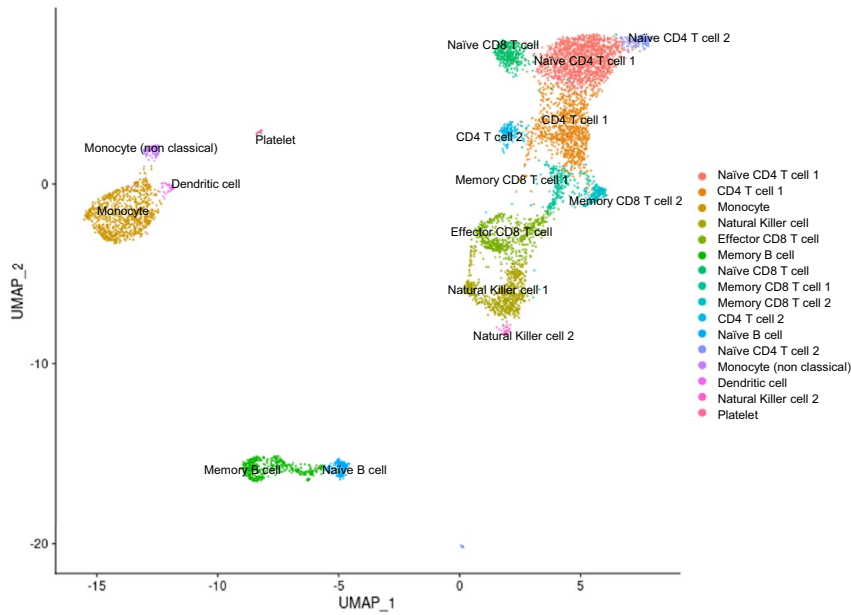
In the subsequent analysis, we focused mainly on the monocyte and NK cell clusters of the PBMCs because the monocyte and NK cell clusters exhibited many upregulated genes in the AIH group. To explore the immune characteristics of patients with AIH, monocytes and NK cells were analyzed in detail. Thereafter, 87 up- and 12 downregulated DEGs were retained in monocytes from AIH PBMCs (Fig. 2c). On the other hand, 101 up- and 15 downregulated DEGs were retained in the NK cells from the AIH PBMCs (Fig. 2d) (Supplemental Table S4).

AIH patient DEGs and their functions in monocytes and NK cells

We further applied GO enrichment analysis to explore the biological processes associated with the DEGs in monocytes from PBMCs (Supplemental Table S5). These terms were related mainly to interferon- γ (IFN- γ)-mediated signaling (GO:0060333), neutrophil degranulation (GO:0043312), and neutrophil activation involved in the immune response (GO:0002283) (Fig. 3a). Interestingly, 15 genes were involved in the two functional categories of neutrophil degranulation and neutrophil activation involved in the immune response, while seven genes were involved in the functional category of IFN- γ -mediated signaling (Supplemental Table S6). The eight neutrophil activation markers, namely *FOLR3*, *HSPA1A*, *MNDA*, *S100A8*, *S100A9*, *S100A12*, *GCA*, and *RETN*, were significantly greater in AIH patients than in HCs (all $p < 0.001$). The eight DEGs that were associated with neutrophil activation genes in monocytes are shown in Fig. 3c. The expression of six IFN- γ -mediated signaling genes, *FCGR1A*, *GBP1*, *HLA-DRB5*, *IFNGR1*, *MT2A*, and *STAT1* (all $p < 0.001$), was significantly greater in the AIH group than in the HCs. The six DEGs that were associated with IFN- γ -mediated signaling genes in monocytes are shown in Fig. 3d.

The 15 GO terms associated with the DEGs in the NK cell cluster were also associated with the immune response (Fig. 3b). The top 10 GO terms included T cell receptor signaling pathway (GO:0050852), antigen processing and presentation of exogenous peptide antigen (GO:0002478), immune response-activating cell surface receptor signaling pathway (GO:0002429), immune response-activating signal transduction (GO:0002757), antigen processing and presentation of exogenous antigen (GO:0019884), antigen processing and presentation of peptide antigen (GO:0048002), response to IFN- γ (GO:0034341), antigen receptor-mediated signaling pathway (GO:0050851), antigen processing and presentation (GO:0019882), and cellular response to IFN- γ (GO:0071346). The two functional categories of antigen processing and presentation and immune response shared nine

a



b

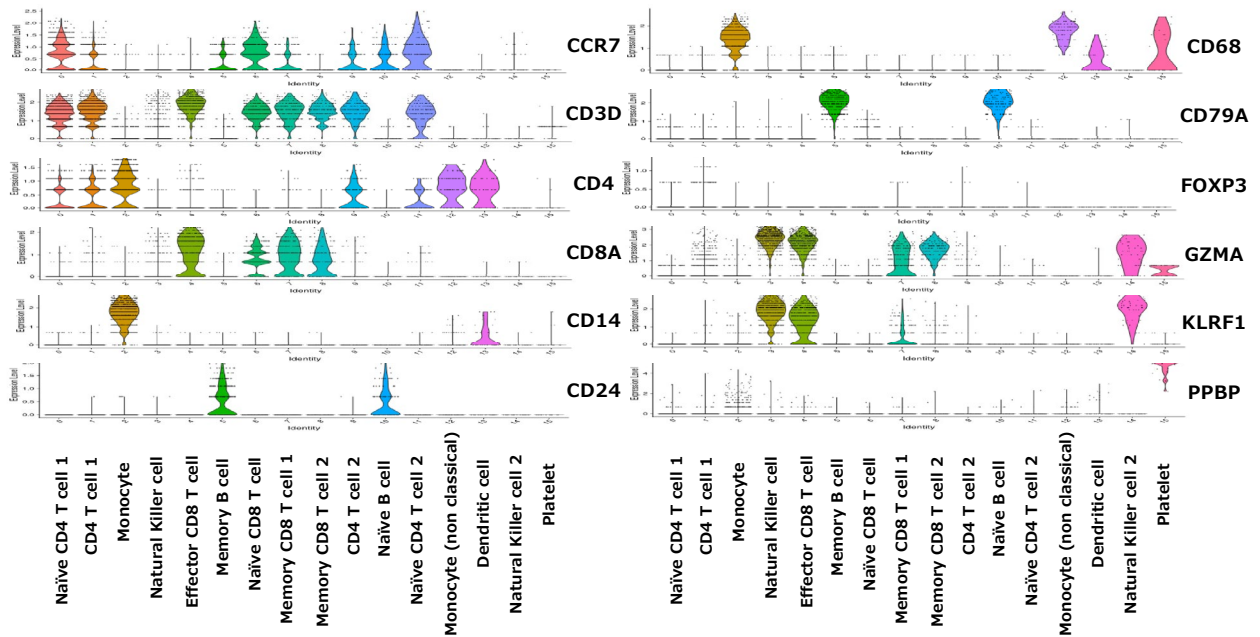


Fig. 1 Cell types identified in peripheral blood mononuclear cells (PBMCs) by uniform manifold approximation and projection (UMAP). Dimensionality reduction was performed using principal component analysis (PCA). The first 50 principal components were used to cluster the cells based on the shared nearest neighbors algorithm (SNN) and visualized in two dimensions by the uniform manifold approximation and projection (UMAP) algorithm. For each cluster, the marker genes were identified using the FindMarkers function as implemented in the Seurat package ($\log_{fc} \text{threshold} > 0.25$ and

$\text{minPct} > 0.2$). Clusters were subsequently assigned to known cell types. **a** Sixteen cell clusters in two samples ($n = 7201$) were clustered into populations as indicated. **b** Violin plot depicting the distributions of cell type marker genes in each cluster using density curves. The width of each violin plot corresponds to the frequency of cells with relevant gene expression levels. **c** Sixteen cell clusters in the HC PBMC samples ($n = 3690$). **d** Sixteen cell clusters in the AIH patient PBMC samples ($n = 3511$). **e** Bar graph of cell clusters in two PBMC samples

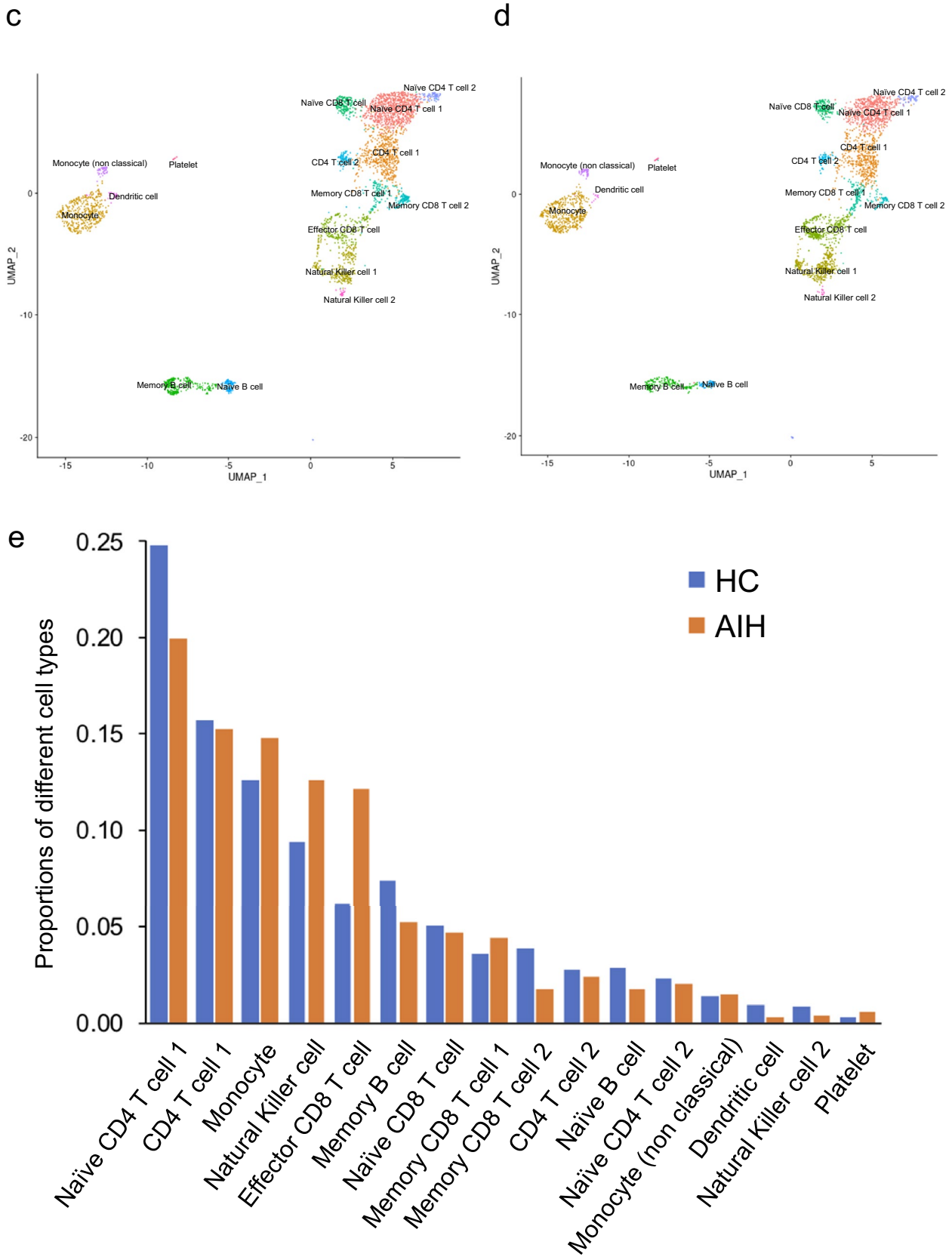


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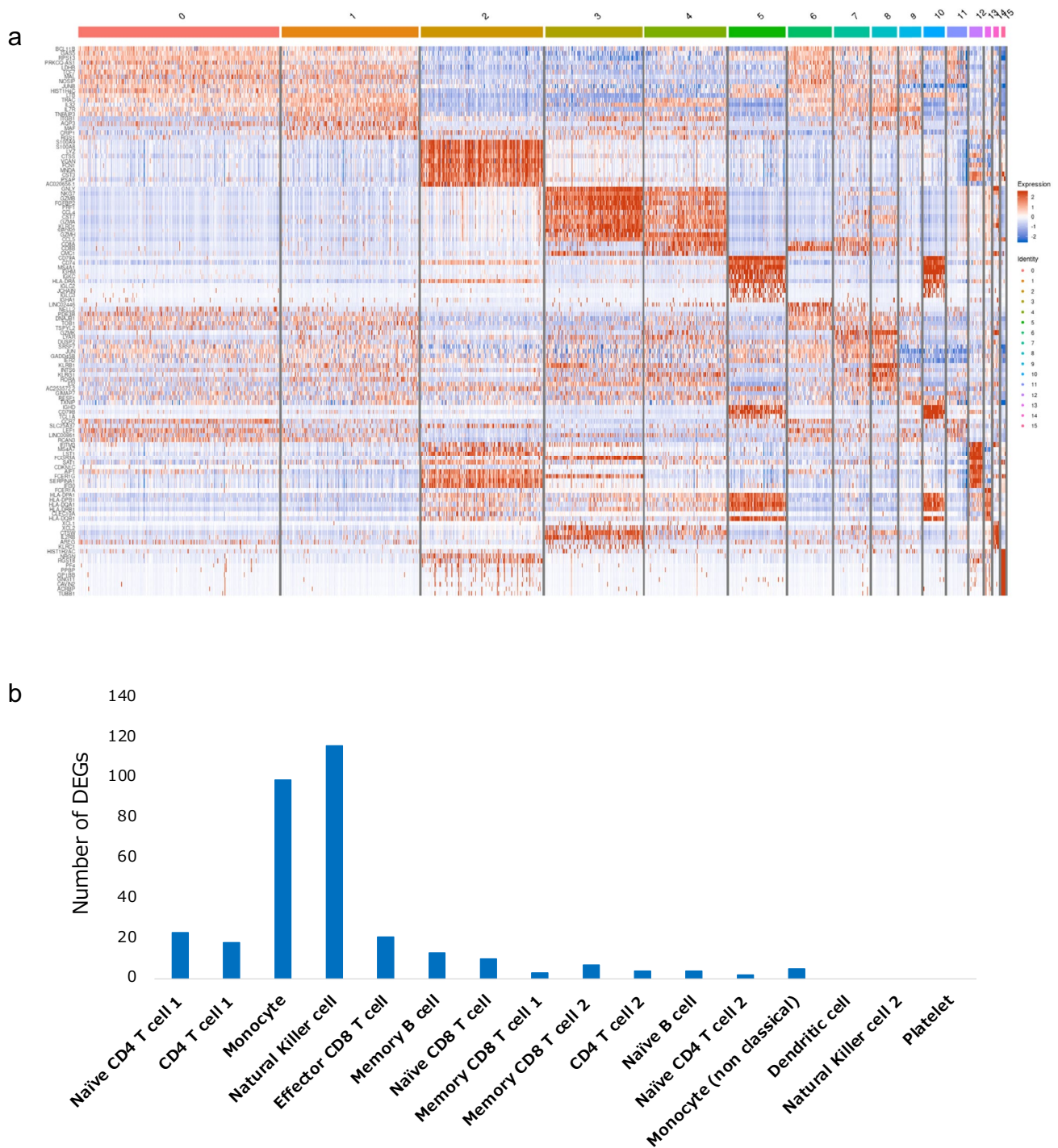


Fig. 2 scRNA-seq analysis of PBMCs from patients with AIH. **a** Heatmap of the top 10 DEGs in each cluster. The top 10 DEGs are labeled in red. Cluster 0: naïve CD4+T cell 1; cluster 1: CD4+T cell 1; cluster 2: monocyte; cluster 3: NK cell 1; cluster 4: effector CD8+T cell; cluster 5: memory B cell; cluster 6: naïve CD8+T cell; cluster 7: memory CD8+T cell 1; cluster 8: memory CD8+T cell 2; cluster 9: CD4+T cell 2; cluster 10: naïve B cell; cluster 11:

naïve CD4+T cell 2; cluster 12: monocyte (nonclassical); cluster 13: dendritic cell; cluster 14: NK cell 2; and cluster 15: platelet. **b** Bar graph of cell cluster differences in the number of DEGs. **c** Volcano plot of DEGs in the monocyte cluster ($|\log_{2}FC| > 0.25$ and adjusted p value < 0.001). **d** Volcano plot of DEGs in the NK cell cluster. Upregulated genes are shown on the right side, and downregulated genes are shown on the left side

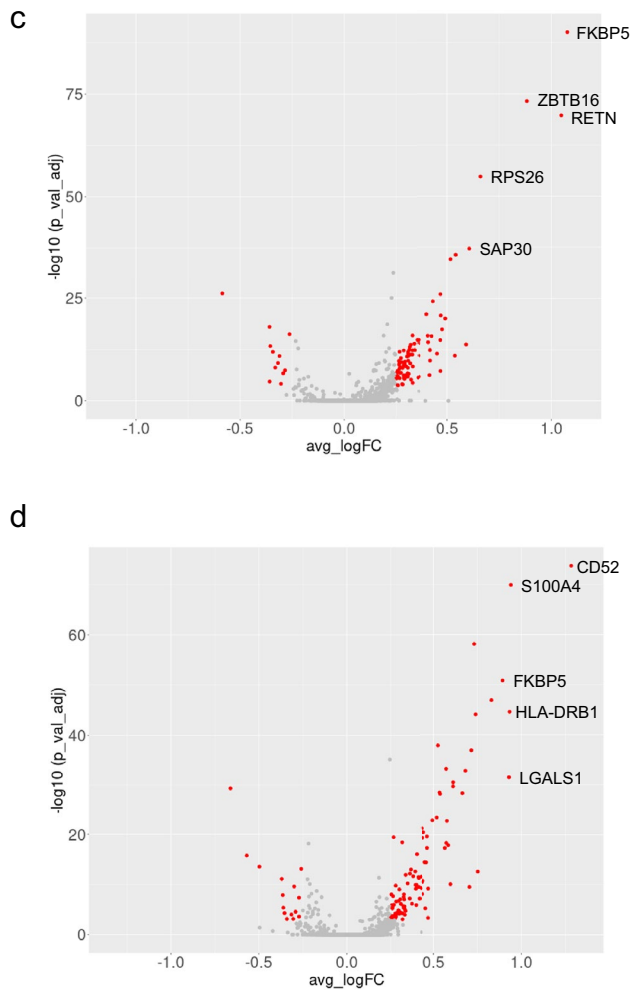


Fig. 2 (continued)

genes, while the functional categories of IFN- γ -mediated signaling and response to IFN- γ shared seven genes (Supplemental Table S7). The expression of seven immune response markers, namely *FCGR3A*, *HLA-DPB1*, *HLA-DRA*, *HLA-DRB1*, *HLA-DRB5*, *LGALS3* and *KLRK1* (all $p < 0.001$), was significantly greater in AIH patients than in HCs. The seven DEGs that represented immune response genes in NK cells are shown in Fig. 3e. The expression of seven IFN- γ -mediated signaling genes, namely *HLA-DPB1*, *HLA-DRA*, *HLA-DRB1*, *HLA-DRB5*, *IFNG*, *SPI100* and *STAT1*, was significantly greater in the AIH group than in the HCs. The seven DEGs that were associated with IFN- γ -mediated signaling genes in NK cells are shown in Fig. 3f.

Discussion

This study is the first to report changes in the PBMCs of AIH patients compared with HCs via scRNA-seq. Although the PCA-based UMAP algorithm was used to

cluster cells with similar expression patterns in the two samples, 87 up- and 12 downregulated DEGs were retained in monocytes, and 101 up- and 15 downregulated DEGs were retained in NK cells from AIH PBMCs ($p < 0.001$ and leverage $|\logFC| > 0.25$). Moreover, the enriched GO terms in the PBMC-derived monocyte and NK cell clusters were related mainly to antigen processing and presentation, IFN- γ -mediated signaling, and neutrophil degranulation and activation. These potential molecular mechanisms may be important targets for AIH treatment.

scRNA-seq can be used to differentiate cell types in complex combined cell populations, promote the recognition of new cell types, and contribute to the understanding of the physiological processes of AIH and the exploration of novel treatment options [31]. Previous reports have shown that a more specific phenotype of memory CD4 + T cells and CD8 + T cells is associated with active AIH based on the data generated from scRNA-seq of circulating autoreactive CD4 + T cells in patients with AIH [18].

NK cells are a key component of the innate immune system and are involved in human autoimmune diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [32, 33]. Under physiological conditions, NK cells constitute 5%–15% of the peripheral blood cell population and are abundant in the liver [34]. A previous report revealed a significant increase in activated effector NK cells in the blood prior to therapy in treatment-naïve AIH patients. Furthermore, the predominant hepatocyte damage in AIH patients seems to originate from effector CD8 + T cell-mediated killing via perforin/granzyme B-mediated cell lysis or the release of IFN- γ [35]. Naïve CD4 + T cells differentiate into regulatory T cells (Tregs) in microenvironments containing various cytokines, where Tregs are induced by TGF- β . However, whether Treg defects in AIH represent the initial causative event leading to immune tolerance breakdown or result from the disease itself remains unclear [36]. In this study, we found that in HCs, the dominant cell clusters were composed of naïve CD4 + T cells, CD4 + T cells, monocytes, NK cells, effector CD8 + T cells, memory B cells, naïve CD8 + T cells, and memory CD8 + T cells. We also observed that the proportions of the main cell clusters were greater in AIH patients than in HCs, with more monocytes, NK cells, and effector CD8 + T cells and fewer naïve CD4 + T cells.

Furthermore, by analyzing the DEGs of immune cells in AIH patients and HCs, we identified *FKBP5* and *RPS26* as the common DEGs shared by the B cell, T cell, monocyte and NK cell clusters. *FKBP5* is the cochaperone of heat shock protein (Hsp) 90 and GR; it can inhibit GR sensitivity to regulate the hypothalamus–pituitary–adrenal (HPA) axis and is highly expressed after stress exposure [37]. A previous report showed that *FKBP5* gene polymorphisms may be associated with depression and glucocorticoid

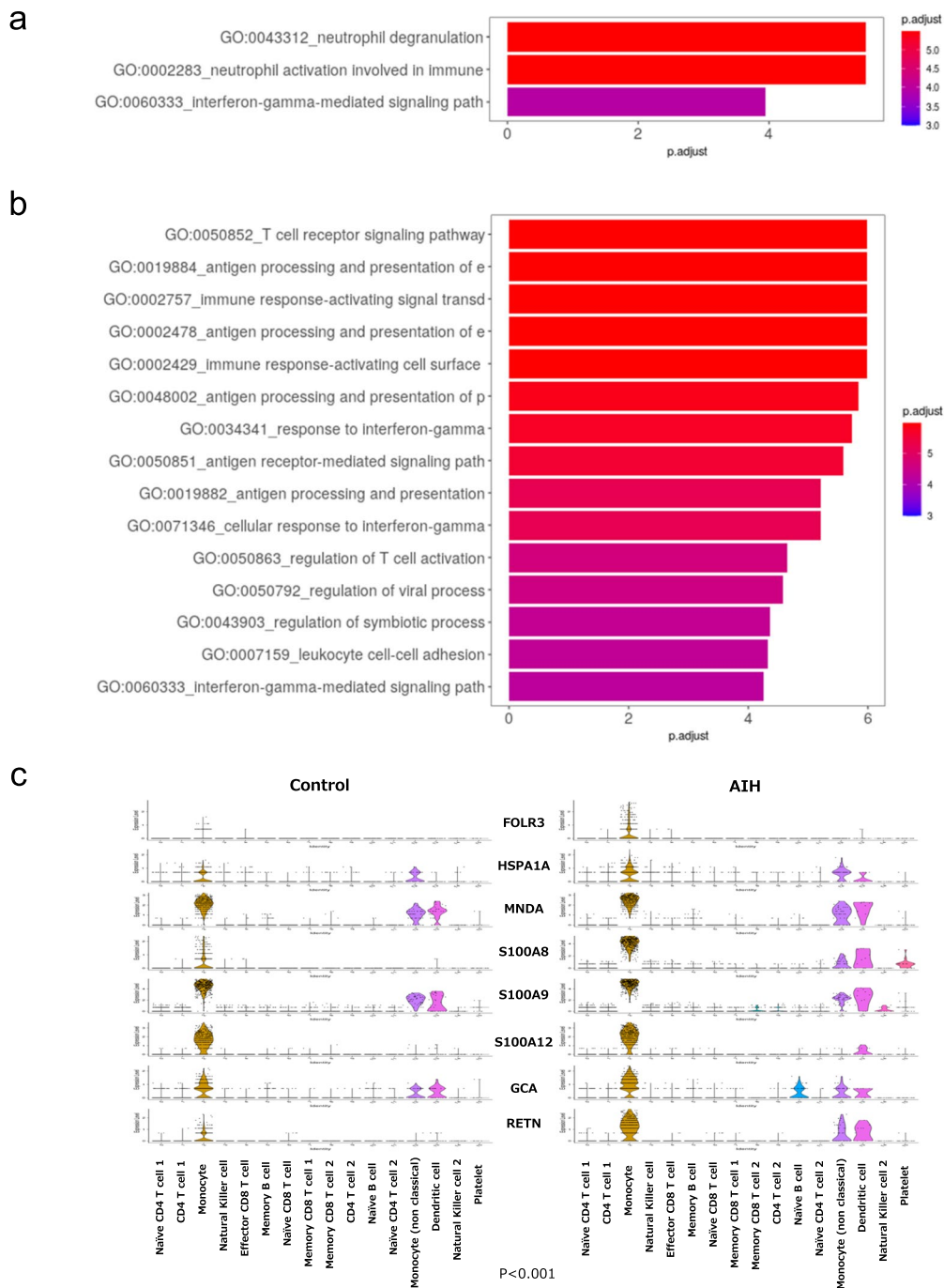


Fig. 3 DEGs in AIH patients and their functions in monocytes and NK cells. **a** GO analysis of DEGs in the monocyte cluster. These terms were related mainly to IFN- γ -mediated signaling (GO:0060333), neutrophil degranulation (GO:0043312) and neutrophil activation involved in the immune response (GO:0002283). **b** GO analysis of DEGs in the NK cell cluster. The top 10 GO terms included T cell receptor signaling pathway (GO:0050852), antigen processing and presentation of exogenous peptide antigen (GO:0002478), immune response-activating cell surface receptor signaling pathway (GO:0002429), immune response-activating signal transduction (GO:0002757), antigen processing and presentation of exogenous antigen (GO:0019884), antigen processing and presentation of peptide antigen (GO:0048002), response to

IFN- γ (GO:0034341), antigen receptor-mediated signaling pathway (GO:0050851), antigen processing and presentation (GO:0019882), and cellular response to IFN- γ (GO:0071346). GO analysis was performed using gene set enrichment analysis (GSEA), and a false discovery rate (FDR) ≤ 0.05 was considered to indicate significant enrichment. **c** The eight DEGs that were associated with neutrophil activation genes in monocytes. **d** The six DEGs that were associated with IFN- γ -mediated signaling genes in monocytes. **e** The seven DEGs that represented immune response genes in NK cells. **f** The seven DEGs that were associated with IFN- γ -mediated signaling genes in NK cells

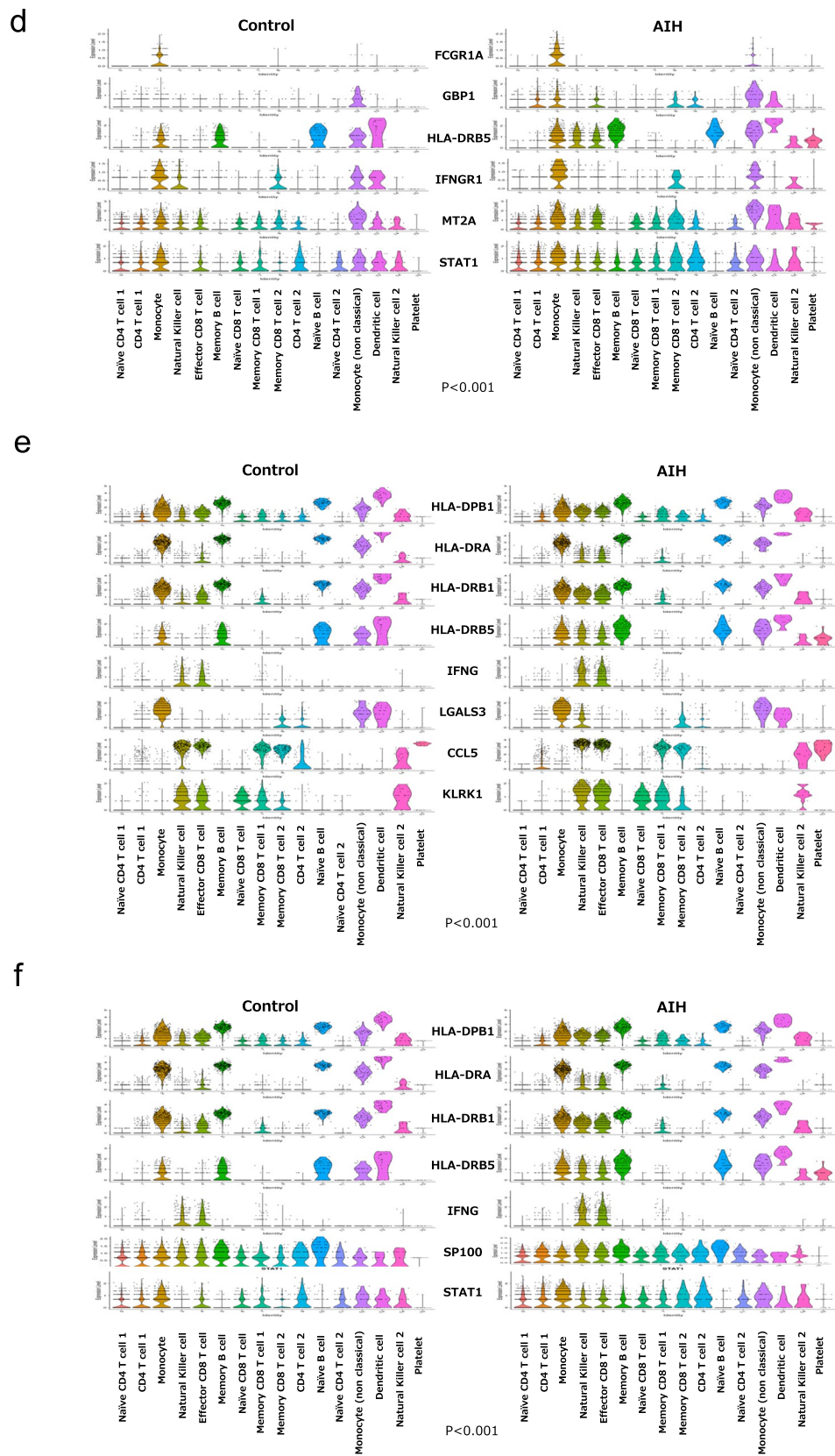


Fig. 3 (continued)

(GC) efficacy in SLE patients [38]. *RPS26* encodes a ribosomal protein that is a component of the 40S subunit. The protein belongs to the S26E family of ribosomal proteins. A previous report showed cell type-specific regulation of the transcript levels of genes associated with several autoimmune diseases in CD4+ and CD8+ T cells, including a trans-acting regulatory locus at chr12q13.2 containing the rs1131017 SNP in the *RPS26* gene [39]. We also identified *TXNIP* and *NEAT1* as common DEGs shared by T cell clusters. *TXNIP* encodes a thioredoxin-binding protein that is a member of the alpha arrestin protein family. Accumulated *TXNIP* activates the NLRP3 inflammasome, consequently inducing the secretion of IL-1b and IL-18 as part of the inflammatory response [40]. *NEAT1* was reported to promote non-alcoholic fatty liver disease progression. Several reports have shown that *NEAT1* is dysregulated and may serve as an emerging novel biomarker and therapeutic target for RA and SLE [41, 42]. These genes may offer a possible new therapy for AIH.

We identified common gene expression patterns in each PBMC-derived cell cluster in AIH patients. These genes are potential therapeutic targets in AIH. Next, we focused on cell clusters with strongly differential gene expression. The activation and expansion of NK cells occurs in the early stages of AIH, as NK cells function in the initial response to liver injury and carry out diverse functions, including cytotoxicity, which may be directed at target cells, and cytokine IFN- γ release, which can promote the maturation of antigen-presenting cells to drive an adaptive immune response [43]. IFN- γ has been identified as the predominant cytokine in AIH patients [44]. Moreover, a polymorphism in the TNF gene promoter has been reported to be associated with type I AIH [45] and the clinical features of AIH patients [46]. In this study, after GO annotation, we observed that the DEGs associated with NK cells were specifically enriched in antigen processing and presentation, immune response, and IFN- γ -mediated signaling. Moreover, monocytes/macrophages are key components of the innate immune system and have numerous immunological functions, including phagocytosis, antigen presentation and cytokine production. Peripheral blood monocytes serve as direct precursors to tissue macrophages. A previous report indicated marked macrophage activation, as shown both by the concentration of CD163 in blood and directly in liver biopsy samples from acute AIH patients [47]. In the present study, the *CD163* gene was upregulated in peripheral blood monocytes, and we believe that CD163+ monocytes contribute to the pathogenesis of AIH-related inflammation (Supplemental Table S3). Furthermore, after GO annotation, we detected that the DEGs of monocytes were specifically enriched in neutrophil degranulation/activation and IFN- γ -mediated signaling.

There is evidence for the role of neutrophils and their distinct LDG (low-density granulocyte) subset in the

development of autoimmune diseases, including AIH [48]. LDGs are neutrophils that, after separation via density gradient centrifugation, remain in the PBMC fraction. More recent studies have also described LDG in AIH [49, 50].

LDG-treated neutrophils strongly express LDG-specific surface markers (CD10, CD15 and CD16). When activated, they can damage endothelial cells and release large amounts of tumor necrosis factor (TNF) and type I and II IFNs [51, 52]. Activated by pathogenic microorganisms or proinflammatory cytokines, LDGs can undergo spontaneous death (i.e., the release of neutrophil extracellular traps (NETs), which is also known as NETosis). The involvement of NETs in AIH as a trigger mechanism has not yet been studied in detail. LDG infiltration, which is likely observed at the early stage of AIH, in combination with the dominant autoantibodies imply the formation of NETs as a component of the disease process and influence an abnormal immune response. Nevertheless, research related to NETs may be helpful in elucidating the mechanism of AIH development and in the elaboration of novel diagnostic and therapeutic strategies [49].

Our study has several limitations. First, the limited sample size of this study prevents solid conclusions from being drawn, as does the decision to pool the samples prior to sequencing, preventing any statistics, heterogeneity, or clinical associations from being derived from the analysis across patients and HCs. However, further expansion of the sample size and further subsequent experimental research are needed. Second, the present study was designed to analyze PBMC characteristics through scRNA-seq, explore cell cluster identification and identify DEGs. However, the expression of related factors in liver tissue was not investigated. Third, the AIH patients in the study were receiving combination therapy comprising prednisolone and azathioprine. Before treatment, an increase in inflammatory cells, especially CD4+ /CD8+ T cells, which are involved in active AIH, is expected. In future, we would like to consider a comparative study of treatment-naïve AIH patients and patients receiving steroid monotherapy.

Conclusions

In conclusion, this study preliminarily explored the immune mechanism of AIH through the use of scRNA-seq technology and explored the relevant molecular markers and major enriched functions through GO analysis. Although the PCA-based UMAP algorithm was used to cluster cells with similar expression patterns in the two samples, 87 up- and 12 downregulated DEGs were retained in monocytes, and 101 up- and 15 downregulated DEGs were retained in NK cells from AIH PBMCs. Moreover, the enriched GO terms in the PBMC-derived monocyte and NK cell clusters

were related mainly to antigen processing and presentation, IFN- γ -mediated signaling and neutrophil degranulation and activation. These markers may be important targets for AIH treatment.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00795-024-00380-5>.

Acknowledgements The authors thank Chikako Saito and Rie Hikichi for their technical assistance.

Author contributions Conceived and designed the experiments: KA and HO. Performed the experiments: KA. Analyzed the data: KA. Contributed materials: KA, NA, TS, YT, AT, MF, and MH. Wrote the paper: KA and HO.

Funding This work was supported by AbbVie Inc. grant funding (No. 1676, No. 2546).

Declarations

Conflict of interest None to declare.

Ethics statement All patients and HCs agreed to blood testing, and written informed consent was obtained. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved for use with the informed consent of participants by the ethics committee of Fukushima Medical University School of Medicine (IRB #2020-200).

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