Single-cell Landscape Reveals the Immune Heterogeneity of Bone Marrow Involvement in Peripheral T-cell lymphoma

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Article

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Abstract

Evaluation of Bone marrow involvement (BMI) in Peripheral T-cell lymphoma (PTCL) patients is quite important for determining the prognosis and the tumor microenvironment of BM in PTCL remains unclear. We collected 11 fresh BM samples from the patients with BMI and performed the single-cell RNA-seq (scRNA-seq) analysis to reveal the immune landscape and genetic variation of different prognosis of PTCL. There were more effector T cells, exhausted lymphocyte cells and inflammatory response in AITL compare with PTCL-NOS. The immune heterogeneity in AITL was associated with prognosis. More specific TCR-T cells enriched was observed in patients with better response to anti-CD30 therapy. RHOA mutation associated neoantigen was observed. In those treated with Chidamide, more CD4 + regulate cells resulted in better response. In the progressive group, T cell enrichment progressed to secondary B cells enrichment was found and later developed diffuse large B cell lymphoma. Moreover, the AITL patient with lymphoma-associated hemophagocytic syndrome contained the precursor exhausted cells with the copy number variation in Chr5. Overall, this study revealed the first single cell landscape depicting BM microenvironment heterogeneity in PTCL with BMI. It is innovative in applying the scRNA-seq technology to investigate the immune heterogeneity and the genetic variation of AITL.

Introduction

Peripheral T-cell lymphoma (PTCL) is a heterogeneous group of non-Hodgkin lymphomas (NHLs), accounting for 10%-15% of NHLs in the western countries, and increased incidence (~20%) of NHLs in Asia countries1–4. PTCL is often characterized by aggressive clinical behavior, poor response to standard chemotherapy, and poor long-term survival5. The World Health Organization (WHO) classification recognizes ~ 30 subtypes of PTCL. The most frequent entities include angioimmunoblastic T-cell lymphoma (AITL), anaplastic large cell lymphoma (ALCL), adult T-cell leukemia/lymphoma (AITL), extranodal NK/T cell lymphoma of nasal type (ENKTL), and the PTCL-not otherwise specified (PTCL-NOS)5. Additional rare PTCLs are mostly extranodal tumors.5.

- The most common entity is AITL characterized by distinct clinicopathological feature, including a dysregulated immune system, such as polyclonal hypergammaglobulinemia, autoimmune response, and immunosuppression resulting in Epstein-Barr virus (EBV) infected B cells neoplasm6–9. The T follicular helper (TFH) cell is the putative original cells in AITL. The expression of TFH markers have been detected in the gene expression profiling (GEP) studies of AITL, which also displays the activation of NF-κB, IL-6, and TGF-β signaling pathways10–13. Moreover, the frequent mutations (e.g. RHOA, TET2, DNMT3A, IDH2) and genomic copy number variation are observed in AITL patients14–23. However, the complete pathobiology of AITL remains poorly defined, and the varied morphology and lack of definitive diagnostic markers still make the diagnosis and classification challenging7,24. With current immunophenotypic and molecular markers, about 30–50% of PTCL cases are not classifiable and are categorized as PTCL-NOS, a heterogeneous group of cases that is difficult to diagnose and treat11,25.
At present, PTCL diagnosis is established by an experienced hematopathologist by identifying morphological and immunophenotypic features\(^{26,27}\). Unfortunately, a large number of PTCL cases do not fit into one particular subtype\(^ {26}\). Gene expression profiling analysis using RNA sequencing (RNA-seq) has been performed for improving the diagnosis of PTCL\(^ {11,28}\). However, RNA-seq is typically performed in bulk, and the data represent an average of gene expression patterns across thousands to millions of cells. This might obscure biologically relevant differences between cells. Single-cell RNA-seq (scRNA-seq) represents an approach to overcome this problem\(^ {29}\). By isolating single cells, capturing their transcripts, and generating sequencing libraries in which the transcripts are mapped to individual cells, scRNA-seq allows assessment of fundamental biological properties of cell populations and biological systems at unprecedented resolution\(^ {29,30}\).

The scRNA-seq is currently one of the most advanced technology in the field of precision medicine, with a very broad application prospect in the disease diagnosis\(^ {30-33}\). This method allows us to quickly and systematically assess the heterogeneity of various types of cells from the patient, identifying the disease-related cells and their genetic and phenotypic characteristics, especially for those few abnormal cells that occur in the early stage of the disease. Additionally, several previous studies have demonstrated that bone marrow (BM) involvement is an independent predictor in patients with PTCL but no studies investigate the interaction between BM involvement and its tumor microenvironment\(^ {24,34-36}\). This study showed the first single cell landscape depicting the tumor microenvironment heterogeneity in the bone marrow of PTCL. Moreover, we also applying the scRNA-seq technology to investigate the immune heterogeneity and the genetic variation at single-cell resolution for the different progress in the treatment of AITL.

**Materials And Methods**

**Patient enrolment**

The PTCLs cases were identified primarily by morphological assessment and the expression of WHO-defiend IHC markers. The pathological diagnosis of PTCL and interpretation of the bone marrow biopsy (BMB) histology results were made on tissue specimens by an experienced hematopathologist. Bone marrow specimens were obtained by trephine biopsy. The presence of lymphoma was based on immunohistochemistry using antibodies against CD3, CD4, CD5, CD7, CD8, CD10, CD20, and cytotoxic molecules. A BM involvement was confirmed by BMB specimens positive for PTCL or the appearance of an initially abnormal BM. We collected 11 fresh bone marrow aspirates samples from the patients with BMI, among them 8 samples are AITL and 3 samples are PTCL-NOS. We also collected 2 samples from the healthy donors as control. This study was approved by the Clinical and Research Ethics Committee of the Guangdong Provincial People's Hospital, Guangzhou, China. All procedures in the present study that involved human participants were performed in accordance with the Declaration of Helsinki.
Single cell collection, library construction and sequencing

Single cell capture was performed using a Chromium Controller instrument (10X Genomics), a highly repeatable, efficient and stable solution for cell characterization and gene expression profiling of thousands to millions of cells (https://www.10xgenomics.com/solutions/single-cell/). Single cells were collected from bone marrow cells, which was isolated by using human bone marrow lymphocyte separation solution. Fresh cells from bone marrow were harvested, washed with 1× PBS and re-suspended at 1× 10⁶ cells per ml in 1× PBS containing 0.04% BSA to minimize cell loss and aggregation following the protocol recommended by 10X Genomics. Cell viability of the samples was analyzed using trypan blue exclusion staining and double fluorescence (AO/PI) staining to ensure more than 80% of live cells. Cellular suspensions were loaded on the Chromium Controller instrument to generate single-cell gel bead-in-emulsions (GEMs) with Chromium Next GEM Single Cell V(D)J Reagent Kits v1.1 (10X Genomics), containing a pool of ~ 750.000 barcodes sampled to separately index the transcriptome of each cell. Thousands of individual cells were isolated into droplets together with gel beads coated with unique primers bearing 10X cell barcodes, unique molecular identifiers (UMI) and poly (dT) sequences. According to the Single Cell V(D)J Reagent Kits protocol, GEM-reverse transcriptions were performed in a Veriti 96-well thermal cycler (Thermo Fisher Scientific). After RT, GEMs were broken and the barcoded single-strand cDNA was cleaned up with DynaBeads MyOne Silane Beads (Thermo Fisher Scientific) and a SPRi select Reagent Kit (Beckman Coulter). Global Amplification of cDNA was achieved using the Veriti 96-well thermal cycler, and the amplified cDNA product was cleaned up with the SPRiSelect Reagent Kit. The scRNA-seq libraries were constructed by using the 5′ Library Kits (part of Single Cell V(D)J Reagent Kits), and the scTCR-seq libraries were constructed by using the V(D)J Enrichment Kits of human T Cell (part of Single Cell V(D)J Reagent Kits). (a) fragmentation, end repair and A-tailing; (b) size selection with SPRi select beads; (c) adaptor ligation; (d) post-ligation cleanup with SPRi select beads; and (e) sample index PCR and final cleanup with SPRi select beads. The constructed libraries were quantified by a Bioanalyzer Agilent 2100 System using a High Sensitivity DNA chip (Agilent), and the quantitative PCR using a KAPA Library Quantification Kit (KAPA Biosystems). Finally, the sequencing libraries were loaded onto Novaseq 6000 (Illumina) platform to generate 2× 150-bp paired-end reads.

scRNA-seq bioinformatics analysis

The single-cell sequencing files were processed using the Cell Ranger Single-Cell Software Suite (v6.0.1) for quality control, sample demultiplexing, barcode processing, and single-cell gene counting (https://support.10xgenomics.com/single-cell-gene-expression/software). The raw base call files of each sample were first demultiplexed into fastq data using the bcl2fastq conversion software. Quality control of the fastq data was performed using FastQC software, and the data were aligned to the Nucleotide Sequence Database (https://www.ncbi.nlm.nih.gov/genbank/) using the basic local alignment search tool (BLAST) to avoid the data distortion caused by the experimental contamination, especially the bacterial infection or contamination. After the initial quality control, the sequences with barcodes and
UMIs of low quality were removed. More than 98% of the clean reads had high quality scores at the Q30 (an error probability for base calling of 0.1%) level in the bases of the barcodes and UMIs.

The raw sequencing data were processed by the Cell Ranger pipeline (v6.0.1, 10X Genomics), including demultiplexing, genome alignment (GRCh38), barcode counting and unique molecular identifier (UMI) counting. The gene-barcode matrix of UMI counts was then analyzed with Seurat (v3.0.2) for quality control, normalization, dimensional reduction, batch effect removal, clustering and visualization \(^{37}\). Any cell with less than 200 genes or more than 20% of mitochondrial UMI counts was filtered out. Cells expressed more than 6000 genes were also excluded. The cell doublets were identified and filtered out by using DoubletFinder (v2.0.2)\(^ {38}\). The single cell TCR-seq data were assembled and analyzed by using the Cell Ranger VDJ pipeline (10X Genomics) and scRepertoire software\(^ {39}\). For clustering the cells, the principal component analysis (PCA) was run on the normalized filtered gene-barcode matrix to reduce the number of feature (gene) dimensions. The first 30 principle components and resolution with 0.8 were adopted to find clusters and the dimensional reduction was performed with the uniform manifold approximation and projection (UMAP).

**Differential expressed genes and enrichment analysis**

After we got the clusters, cluster-specific genes named "markers" were calculated with Seurat (v3.0.2) and cell types were annotated by top markers in each cluster. Canonical cell type genes were also used in cell annotation, like CD79A/B for B cells, CD3 for T cells, LYZ/AIF1 for Myeloid cells, MZB1/IGHG1 for plasma cells, LILRA4/IRF8 for pDCs, GYPC/GYPA for Erythrocytes, MKI67 and STMN1 for cycling or proliferation cells. For cell annotation of detailed cluster in each cell type like T or Myeloid cells, top markers were used to name them. Significant expressed genes were selected by a cutoff with 0.5 for avg_logFC and 0.05 for p_val_adj. Significant expressed genes were used for GO or KEGG enrichment analysis using R package clusterProfiler(v3.12.0)\(^ {40}\). To assess the exhaustion or pro-inflammatory states in cell clusters, function "AddModuleScore" in Seurat (v3.0.2) were used to calculate the signature score based on given genesets. For exhaustion score, common genes like PDCD1, LAG3, TIGIT, CTLA4, TOX, HAVCR2 were used. For pro-inflammatory score, related cytokine genes were selected: i.e. IL1B, TNF, CCL2, CCL3, CCL7, CCL8, CCL13, CCL17, CCL22.

Group-enrichment analysis were performed on ratio of observed to expected following the published article\(^ {41}\). Briefly, based on cell count of each group in given clusters(observed), a contingency table was constructed to calculate the expect cell count (like statistics in chisq.test). Ratio of observed cell count to expected cell count were used to classify the group-enrichment, ratio > 1 was regarded as "enrichment", otherwise "depletion".

**Single cell SNP/CNV calling and Neoantigen/Epitopes prediction**
We used VarTrix to identify the SNP status of RHOA in each cell. VarTrix would take a set of previously defined variant calls and use that to identify those variants in the single cell. RHOA SNPs were selected from dbSNP database. Based on VarTrix results, we classified the “alt/alt” and “alt/ref” as the final mutation. To find the aberrant T cells with clonal large-scale chromosomal copy number variations, we used the R package inferCNV(v1.0.4) to infer the genetic profiles of each cell based on the average expression of large gene sets (https://github.com/broadinstitute/inferCNV). As T cells from normal/healthy donors were mainly distributed in CD4 + naive T cells and CD8 + naive T cells, these two naive T cell clusters were regarded as normal/reference. Other parameters were set as default.

Whole exome sequencing (WES) was performed and variants were called based on WES data. After we got the final mutation result, these somatic VCF files were analyzed by pVACseq limited to MHC class I alleles(HLA-A, HLA-B, HLA-C). In brief, HLA typing was performed on raw fastq data using OptiType(v1.3.3). Based on the HLA alleles, module pVACseq from pVACtools(v1.0.0) was used to predict the peptides binding to these alleles. To get more specific peptides, pVACseq used the median binding score of many prediction algorithms as final binding score. Finally, epitopes with binding score lower than 500 were retained.

**Data availability**

The processed sequencing data generated in this study have been deposited in the Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra/) under a Bioproject accession code PRJNA809564.

**Results**

**Cellular heterogeneity of AITL compared to PTCL-NOS.**

The PTCL cases were identified primarily by morphological assessment and the expression of WHO-defined IHC markers. The pathological diagnosis of PTCL and interpretation of the bone marrow biopsy (BMB) histology results were made on tissue specimens by an experienced hematopathologist. We collected 11 fresh bone marrow aspirates samples from the patients with BMI, including 8 samples of AITL and 3 samples of PTCL-NOS. We also collected 2 samples from the healthy donors as control.

Based on the scRNA-seq profiles, we clustered totally 60170 cells of different type across samples by using Seurat software (v3.0.2) and visualized them in two-dimensional space (Fig. 1A and B). The cell clusters were annotated with SingleR software (v1.0.1) and refined with expression of canonical marker genes (Fig. 1C and D). Major cell types comprising the bone marrow cells were well captured for scRNA-seq, including T cells (CD3D, CD3E, CD3G), natural killer (NK) cells (NCAM1 or CD56, KLRB1, NKG7), B cells (CD79A, CD79B, MS4A1), myeloid cells (CD1C) and plasmacytoid dendritic cells (pDCs) (LILRA4). There were also some residual erythrocytes (HBB) and megakaryocytes (PPBP) mixed in the bone marrow (Supplementary Fig. 1). We pooled the bone marrow cells by conditions and found that, compared with healthy controls, the AITL patients showed a decreased percentage of B cells, while an
increased percentage of myeloid cells and NK cells (Fig. 1B). These observations were in line with PTCL-NOS, suggesting that scRNA-seq recovered key immune alterations of PTCLs (Supplementary Fig. 2). However, the pro-inflammatory response in AITL was more active than that in PTCL-NOS and healthy control (Fig. 1E, both p value < 10^{-16}). As lymphoma has been associated with cytotoxic lymphocyte exhaustion, we profiled the expression of genes encoding canonical exhaustion markers by T and NK cells. The significant exhaustion of T cells was only found in patients with AITL (Fig. 1F, both p value < 10^{-16}). However, the NK cells from all samples with AITL or PTCL-NOS appeared exhausted based on expression of LAG3, PDCD1, TIGIT and HAVCR2 (Fig. 1G).

In addition to these common features in PTCLs compared with healthy controls, there are some unique characteristics for AITL or PTCL-NOS. The immune alterations between AITL and PTCL-NOS are mainly concentrated in lymphocyte and myeloid cells. We separated these cells for a more detailed and comprehensive analysis. In the cluster of 28463 lymphocytes, there were 11 sub-clusters named with CD4 + memory, CD4 + naive, CD4 + Treg, CD8 + effector GZMB+, CD8 + effector GZMK+, CD8 + effector Memory, CD8 + effector CMC1+, CD8 + naive, Cycling, Cycling PCLAF + and precursor-exhausted cells (Fig. 2A-D). We identified the proliferative lymphocytes cells that appeared to be increased in the samples of AITL (10%) and PTCL-NOS (21%) compare with healthy control. The proportion of CD4 + Naive and CD8 + Naive cells were dramatically decreased (AITL: CD4 + 38–10%, CD8 + 41–11%; PTCL-NOS: CD4 + 38–17%, CD8 + 41–8%) in the patients compared with healthy control. These naive cells developed into other effector cells, regulator cells and memory cells. The CD8 + effector cells (GZMB + and GZMK+) and CD4 + regulator cells (FOXP3+) were enriched in the patients of AITL compared with PTCL-NOS containing high proportion of CD8 + CMC1 + effector cells (28%). There was no significant evidence of CD4 + T cell and CD8 + T cell exhaustion in patients. Moreover, we observed an increased level of precursor exhausted T (Texp) cells enriched in AITL patients specifically (Fig. 1D,).

Based on the differentially expressed gene (DEG) signatures, we identified relatively specific genes that were highly expressed in AITL patients were enriched in T cell activation and regulation panels (Supplementary Fig. 3). The top enriched GO terms of biologic process also included the response to interferon and pro-inflammatory cytokines (Fig. 2E). Conversely, the relatively specific genes that were highly expressed in PTCL-NOS patients were mainly enriched in the neutrophil activation (Fig. 2F), suggesting the cross-talk between T cells and neutrophil cells in PTCL-NOS.

In the cluster of 21645 myeloid cells, there were 11 sub-clusters named with CD14 + monocyte, VCAN + CD14 + monocyte, IL1B + CD14 + monocyte, CD16 + monocyte, FCGR3B + neutrophil, DEFA3 + neutrophil, IFITM2 + neutrophil, myeloid dendritic cells (mDC) and proliferative myeloid cells (MKI67) (Fig. 3A-C and Supplementary Fig. 4). We identified more activated monocyte cells enriched in the patients of AITL and more neutrophil cells enriched in the patients of PTCL-NOS (Fig. 3D). Theses neutrophil cells may be activated by the T cells with highly expressed genes enriched in the GO term of neutrophil activation in the patients of PTCL-NOS. For the patients of AITL, the relatively specific genes that were highly expressed in myeloid cells were mainly enriched in the Toll-like receptor, NF-kappa B, or TNF signaling pathways (Fig. 3E and F), which were the canonical pathway enriched in AITL compared with other PTCL
(Fig. 3E and F). In addition, the HLA class II upregulation in the monocyte cells were enriched in the patients of AITL (Fig. 3E).

**Immune heterogeneity of bone marrow involvement in AITL under different conditions**

The immune heterogeneity is not only related to the development of different cell types with lymphoma, but also could affect the progresses of disease. After treatment, the progresses of AITL patients collected were in different conditions. We divided these patients into four groups based on the prognosis after the treatment: the response group under Anti-CD30 antibody-based therapy (Anti-CD30, n = 3); the response group under Chidamide treatment (Chidamide, n = 2); the progressed group developed into the diffuse large B cell lymphoma (DLBCL, n = 2); the progressed group developed into the lymphoma-associated hemophagocytic syndrome (LAHS, n = 1). Further analyses showed the immune heterogeneity associated with these different prognoses after treatment. For the lymphocytes, the proportion of different subtypes varies greatly (Fig. 4A-C). In the response group, we found more CD8+ effector cells were enriched in the Anti-CD30 group, and more CD4+ regulatory and memory cells were enriched in the Chidamide group. In the progressed group, more plasma cells and CD4 cells with the expression of CD79, a maker gene of B cells, were enriched in the DLBCL group, indicating the early signs of secondary B-cell lymphoma (supplementary Fig. 5). Moreover, the precursor exhausted cells were found enriched in the progressed group of LAHS (Fig. 4D).

Based on the DEG signatures, we identified relatively specific genes that were highly expressed in the response group of Anti-CD30 were enriched in T cell activation and cytotoxicity. In the response group of Chidamide, the relatively specific genes highly expressed were enriched in T cell regulation and proliferation. Compared with the progressed group, the immune response was more active in the response group (Fig. 4E).

For the myeloid cells, more neutrophil cells and proliferative cells were enriched in the response group of Anti-CD30 (Fig. 5A, B, C and D), while more monocyte cells and IFITM2+ neutrophil cells were enriched in the response group of Chidamide (Fig. 5D). Compared with other progressed group, the IL1B+CD14+ monocyte cells in the group of Chidamide and the DEFA3+ neutrophil cells in the group of Anti-CD30 were more prominent in the proportion. Compared with the progressed group, the immune response of myeloid cells were more active in the response group. However, the pro-inflammatory response caused by the myeloid cells were enriched in the progressed group of LAHS (Fig. 5E).

**Genetic variation associated with the BMI progress of AITL after the treatment**

Besides the immune heterogeneity, the genetic variations associated the progress of AITL were found in the bone marrow involvement. In the group Anti-CD30, the RHOA mutation was found enriched (Fig. 6A). We performed whole exome sequencing (WES) and single-cell T cell receptor sequencing (scTCR-seq) for confirming the RHOA mutation in these samples. The HLA typing and neoantigen prediction were conducted based on these sequence data (supplementary Fig. 6). We found that there was indeed the neoantigen of RHOA mutation peptide as expected (Fig. 6B). The mutation-associated neoantigens
(MANA) may cause the enrichment of specific TCR-T cells. Based on this hypothesis, the single-cell immune profiling of these samples were obtained. We found the enrichment of specific TCR-T cells in the group of Anti-CD30 compared with other AITLs, which is beneficial for the immunotherapy (Fig. 6C). For the response group under Chidamide treatment, we found the clonal expansion in CD4 T cells with the same TCR sequence.

However, not all genetic variants leading to the better treatment outcome. Based on the scRNA-seq data, we found that the copy number variation happened in the group LAHS with chromosome 5 (chr5) gain and enriched in the precursor exhausted cells (Fig. 6D and E). This group is characterized by lymphoma-associated hemophagocytic syndrome with aggressive clinical behavior. The examination of chr5 genes in cases with GEP showed that 95 genes were significantly upregulated in the cases with chr5 gain, including IL4, IL13, and MAPK9, which affect cell cycle regulation and T-cell differentiation. Many of these genes related to cell cycle and T-cell activation were also significantly up-regulated in cases with chr5 gain, suggesting the potential connection with the lymphoma-associated hemophagocytic syndrome.

**Discussion**

PTCL is a heterogeneous group of non-Hodgkin lymphoma with lymphoproliferative disorders. It is often characterized by aggressive clinical behavior with bone marrow involvement. Evaluation of BM involvement in PTCL patients is essential to determine their prognosis. However, PTCL patients usually show highly different distributions of lymphoma lesions, which make the interpretation of the results of a single BM biopsy more complicated and the tumor microenvironment of bone marrow in PTCL patients remains unclear. Until now, there was not any tumor microenvironment study analyzing a sizeable cohort that comprised solely PTCL patients with bone marrow involvement. This study aimed to investigate the immune landscape and the genetic variation at single-cell resolution at different progress of PTCL.

This study is the first single cell landscape depicting the tumor microenvironment heterogeneity in the bone marrow of PTCL. Although the proportion of different cell types in the patients of PTCL were not changed very much compared with the healthy control, the transcriptome of these cells from the patients were significantly changed. Most of the naive cells were under cell differentiation, and the exhausted NK cells was found both in AITL and PTCL-NOS, indicating the common immune alteration of AITL and PTCL-NOS. The percentage of exhausted NK cells was increased in the PTCL patients, suggesting that these exhausted NK cells may play a role in the progress of PTCL. Previous report has described a novel immune evasion strategy in classical Hodgkin lymphoma and diffuse large B-cell lymphoma mediated by the expansion of an exhausted NK cell population. In this study, the immune evasion mediated by the exhausted NK cells was also found in PTCL.

We found more inflammatory response and exhausted lymphocyte cells enriched in AITL compare with PTCL-NOS, suggesting the tumor microenvironment of bone marrow in AITL is more complicated.
compared with that of PTCL-NOS. After the treatment, there are more conditions in the patients of AITL. The immune heterogeneity maybe associated with these different prognoses after treatment. We divided all AITL patients into four group by conditions: the response group under Anti-CD30 treatment, the response group under Chidamide treatment, the progressed group developed the diffuse large B cell lymphoma, and the progressed group developed the lymphoma-associated hemophagocytic syndrome. The immune alterations occurred in theses group indicating the immune response was more active in the response group compared with the progressed group. In the response group, we found more CD8+ effector cells were enriched in the Anti-CD30 group, and more CD4+ regulatory and memory cells were enriched in the Chidamide group. These features can be used to guide the treatment of AITL.

Besides the unique immune alteration features, we found that the genetic variation is associated with the response group under Anti-CD30 treatment. More specific TCR-T cells were found enriched in the patients with better response in the group under Anti-CD30 treatment. Further analysis showed the RHOA mutation associated neoantigen was observed in these patients, implying a new target for the immunotherapy of these patients with neoantigen and specific TCR-T cells.

For the progressed group, the unique immune alteration features were also found. More plasma cells and CD4 cells with the expression of CD79 were enriched in the DLBCL group, indicating the early signs of secondary B-cell lymphoma. More myeloid cells with the pro-inflammatory response and precursor exhausted cells were enriched in the group LAHS. Moreover, the AITL patients with lymphoma-associated hemophagocytic syndrome contain the precursor exhausted cells with the copy number variation in Chr5. There may be a certain correlation between these features and the aggressive clinical behavior.

In conclusion, this is the first single cell landscape depicting the immune heterogeneity of bone marrow from the patients with PTCL. Moreover, it is innovative in applying the scRNA-seq technology to investigate the immune heterogeneity and the genetic variation at single cell solution for the different progress in the treatment of AITL. However, more cases are needed for further confirming these association, and explore the implications.

**Declarations**

**Acknowledgments**

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**Author Contributions**
JL, BJX, LXC performed bioinformatics analysis, interpreted data and wrote the manuscript; XMJ collected samples, clinical information, interpreted data and wrote the manuscript; ZHX and LTL performed bioinformatics analysis and interpreted data; WYL, HZ and SJZ designed and supervised the study and wrote the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors declared that they have no conflicts of interest to this work.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article.

Data availability

The processed sequencing data generated in this study have been deposited in the Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra/) under a Bioproject accession code PRJNA809564.

Ethics approval and consent to participate

This study was approved by Guangdong Provincial People's Hospital and the Institutional Review Board/Ethics Committee of Guangdong Provincial People's Hospital. All patients provided informed written consent to participate prior to sample collection.

Consent to publication

Written consent was collected from each patient for future data publication.

References


Figures
Figure 1

Major cell types comprising the bone marrow cells captured by scRNA-seq.

(A) The dimensional reduction is performed with the uniform manifold approximation and projection (UMAP). Each dot represents a cell, which is colored according to cell type. The cells are pooled across all patients and separated by conditions: AITL, angioimmunoblastic T-cell lymphoma (AITL); PTCL-NOS,
PTCL—not otherwise specified (PTCL-NOS); Normal, healthy controls. (B) Percentages of major cell types under each condition are shown on the pie chart. pDC, plasmacytoid dendritic cells. (C) The violin plots showing the expression of cell-specific markers. (D) Heatmap of cell-specific marker expression in each major cell type. (E) Pro-inflammatory score of all cells in each condition group. ***P<0.001. (F) Exhausted score of T cells in each condition group. ***P<0.001. (G) Exhausted score of NK cells in each condition group. *P<0.05, **P<0.01.
Figure 2

Major subtypes comprising the T cells captured by scRNA-seq from bone marrow.

(A) The dimensional reduction is performed with the uniform manifold approximation and projection (UMAP). Each dot represents a cell, which is colored according to cell type. The cells are pooled across all patients and separated by conditions. (B) Percentages of major cell types under each condition are shown on the pie chart. (C) The violin plots showing the expression of cell-specific markers. (D) Dot plot showing the cell type preference measured by Ro/e (ratio of observed cell count to expected cell count) in each condition. Ro/e >1 was regarded as enrichment. (E) Dot plot of GO Biological Process enrichment of significant increased genes in T cells from AITL compared to PTCL-NOS. (F) Dot plot of GO Biological Process enrichment of significant increased genes in T cells from PTCL-NOS compared to AITL.
Figure 3

Major subtypes comprising the myeloid cells captured by scRNA-seq from bone marrow.

(A) The dimensional reduction is performed with the uniform manifold approximation and projection (UMAP). (B) Each dot represents a cell, which is colored according to cell type. The cells are pooled across all patients and separated by conditions: AITL, angioimmunoblastic T-cell lymphoma (AITL);
PTCL-NOS, PTCL-not otherwise specified (PTCL-NOS); Normal, healthy controls. (C) Percentages of major cell types under each condition are shown on the pie chart. (D) The violin plots showing the expression of specific markers in each cluster. (E) Dot plot showing the cell type preference measured by Ro/e (ratio of observed cell count to expected cell count) in each condition. Ro/e >1 was regarded as enrichment. (F) Heatmap of top 5 markers expressed in each cluster. (G) Dot plot of GO Biological Process enrichment of significant increased genes in myeloid cells from AITL compared to PTCL-NOS.
Figure 4

Major subclusters comprising the T cells captured by scRNA-seq from the bone marrow withAITL in different progress.

(A) The dimensional reduction is performed with the uniform manifold approximation and projection (UMAP). Each dot represents a cell, which is colored according to cell type. The cells are pooled across all patients and separated by conditions: Anti-CD30, the response group under Anti-CD30 treatment (Anti-CD30); Chidamide, the response group under Chidamide treatment; DLBCL, the progressed group developed into the diffuse large B cell lymphoma; LAHS, the progressed group developed into the lymphoma-associated hemophagocytic syndrome. (B) Percentages of major cell types under each condition are shown on the bar chart. (C) Heatmap of top 10 markers expressed in each group. (D) Dot plot showing the cell type preference measured by Ro/e (ratio of observed cell count to expected cell count) in each group. Ro/e >1 was regarded as enrichment. (E) Dot plot showing compared GO Biological Process enrichment of group-specific markers between Anti-CD30, Chidamide, DLBCL and LAHS group.
Figure 5

Major subclusters comprising the myeloid cells captured by scRNA-seq from the bone marrow with AITL in different progress.

(A) The dimensional reduction is performed with the uniform manifold approximation and projection (UMAP). Each dot represents a cell, which is colored according to cell type. The cells are pooled across all
patients and separated by conditions: Anti-CD30, the response group under Anti-CD30 treatment (Anti-CD30); Chidamide, the response group under Chidamide treatment; DLBCL, the progressed group developed into the diffuse large B cell lymphoma; LAHS, the progressed group developed into the lymphoma-associated hemophagocytic syndrome. (B) Percentages of major cell types under each condition are shown on the bar chart. (C) Heatmap of top 10 markers expressed in each group. (D) Dot plot showing the cell type preference measured by Ro/e (ratio of observed cell count to expected cell count) in each group. Ro/e >1 was regarded as enrichment. (E) Dot plot showing compared GO Biological Process enrichment of group-specific markers between Anti-CD30, Chidamide, DLBCL and LAHS group.
Figure 6

Genetic variation identified at single-cell resolution associated with different progresses during the treatment of AITL.

(A) UMAP plot showing the distribution of cells with RHOA-G17V mutation. RHOA-G17V mutation was verified by Vartrix with RHOA gene locus type “Alteration/Reference” and “Alteration/Alteration” which
lead to G17V variant. (B) Neoantigen prediction result showing a special epitope for RHOA-G17V. (C) UMAP plot showing the distribution of cells with top proportion of TCR in each group. TRA or TRB sequences of CDR3s Amino Acid are shown in legend. (D) Heatmap of InferCNV result showing a CNV gain enrichment in Chr5. (E) UMAP plot showing the distribution of cells with chr5 CNV gain.

Supplementary Files

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- SupplementaryFigure1XXXXXX.pdf