Single-cell RNA sequencing to explore the immune process difference between Kawasaki disease and febrile patients

Qiu-ping Lin[1], Li-jian Xie[1,2]*, Guang Li[3], Ru-lan Liu[3], Zhen Wang[4]

[1]Department of Cardiology, Shanghai Children′s Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [2]Department of Pediatrics, JinShan Hospital, Fudan University, Shanghai, China, [3]Daozhi Precision Medicine Technology (Shanghai) Co. Ltd, Shanghai, China, [4]Shanghai Institute of Nutrition and Health, University of Chinese Academy of Sciences, Chinese Academy of Sciences, Shanghai, China.

***Correspondence:** Xie Lijian, Email: naijileix@aliyun.com

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Objective: This present study was designed to profile the characteristics of Kawasaki disease (KD) and febrile patients' peripheral blood mononuclear cells (PBMC) to find specific markers for KD diagnosis and to explore the etiology of KD.
Methods: PBMC was collected from two KD patients before and after therapy and two febrile patients, which were used to single-cell RNA sequencing (scRNA-seq) for cell clusters identification. Differently expressed gene (DEG) determination and gene set enrichment analysis (GSEA) were used to explore the biological function.
Results: The dynamic changes were detected in immune cells of KD patients before and after therapy and febrile patients. GSEA revealed that T cells in acute KD were mainly enriched in cell cycle related pathways relative to therapy. Comparing with febrile patients, the percentage of CD8+T cells decreased in acute KD. GSEA revealed that immune cells were enriched in interferon alpha response pathway in acute KD.
Conclusion: The abnormal immune cell development, proliferation and differentiation may be the potential mechanism for the immune disorder in acute KD. And the hygiene hypothesis may be the potential etiology of KD. T cells, especially the CD8+T cells and the interferon response may be the key point to distinguish KD from other febrile disease.

Introduction

Kawasaki disease (KD) is an acute systemic vasculitis that occurs predominantly in children under 5 years of age, which is the most common cause of acquired heart disease in children in developed countries, with coronary artery lesion (CAL) as a

major complication. High-dose intravenous immunoglobulin (IVIG) combined with aspirin is the standard treatment for KD(1). The etiology of KD reminds unknown. Although there exist multiple hypotheses, including the infection hypothesis, the autoimmune hypothesis, and the environmental toxin exposure hypothesis, none of them have been proven(2). The most widely accepted etiologic hypothesis suggested that KD was caused by a kind of prevalent pathogen, which infected children with a genetic susceptibility(3). In recent years, the hygiene hypothesis has been proposed as a new theory to explain the etiology of KD. The core of the hypothesis is that children are exposed to too few microorganisms in the environment and lack of immune exercise, resulting in delayed B cell development and immune deficiency, thus triggering KD. Socioeconomic development accompanied by increased incidence of KD, protective effects of vaccination and breast milk, high IgE levels and low IgG levels in KD patients and high incidence at low immunoglobulin levels, all of these evidences support the hypothesis(4). But the hypothesis has not been confirmed at the cellular level.

The diagnosis of KD currently relies on clinical features, including fever, rash, conjunctivitis, changes in the oral mucosa and extremities and cervical lymphadenopathy(1). Several febrile diseases have similar clinical manifestations to KD, such as scarlet fever, EBV infection, juvenile idiopathic arthritis, measles and adenovirus infection, leading to KD difficulties in diagnosis and delays in treatment(5). In addition, 15.0% to 36.2% of children with KD have atypical clinical signs, which may lead to delays in IVIG treatment while waiting to rule out other febrile illnesses(6). Although many diagnostic tests exist for differentiating KD from other febrile diseases, they are mostly based on clinical features, laboratory variables and gene expression signatures(5, 7, 8). The diagnostic process is cumbersome and complicated. There is a lack of simple and efficient variables to distinguish KD from other febrile diseases.

During the acute phase of KD, it can find that the proportion and abundance of innate immune cells such as peripheral neutrophils, monocytes and macrophages increased significantly, while B and T cells were significantly decrease(9-12). Studies have shown that significant immune disorder occur in the acute phase of KD including changes in immune cells, cytokine cascade stimulation, and endothelial cell activation, demonstrating that activation of immune system is an important feature of KD(13). Single-cell transcriptomic sequencing (scRNA-seq) analyzes the expression profile of the cellular transcriptome at the level of individual cells, allowing the quantification of gene expression, functional enrichment, and metabolic pathways analysis of mRNA in single cell, providing a high-dimensional assessment of the transcriptional status of cells. It provides a more effective tool to distinguish KD from other febrile diseases, thus screening more specific variables for the early diagnosis of KD, reducing the occurrence of CAL and further providing new insights into the etiology of KD and the therapeutic mechanism of IVIG.

In this study, we profile peripheral blood mononuclear cells (PBMCs) isolated from acute KD patients and febrile patients at single-cell resolution. We found a significant immune disorder in the acute phase of KD, and the gene set enrichment

analysis (GSEA) showed it was concentrated in the cell cycle related pathway, suggesting that abnormalities in immune cell development, proliferation and differentiation may be the potential mechanism for the immune disorder occurring in KD. Comparing with febrile patients, GSEA of all kinds of immune cells in acute KD were enriched in the interferon-alpha pathway, providing a new insight for the differentiation of KD from other febrile diseases.

Materials and methods

Enrollment of participants and collection of samples

All donors were recruited from Shanghai Children's Hospital. The diagnosis of KD was made by using the criteria proposed by the American Heart Association(1), including fever of unknown etiology lasting for ≥5 days, and the presence of five associated symptoms (conjunctivitis, oral changes, extremity changes, rash, cervical lymphadenopathy). Complete KD was diagnosed if at least four of the symptoms were fulfifilled, otherwise incomplete KD. The KD patients received high-dose IVIG (1 g/kg per day) for two consecutive days combined with oral aspirin (30 mg/kg per day) after diagnosis with KD. CALs were regularly monitored by two-dimensional echocardiography. One of KD patient was a 5.6 years old boy and was diagnosed with complete KD. The other one was a 5.4 years old girl and was diagnosed with incomplete KD. As for the febrile patients, one was a 4.8 years old boy and one was a 5.1 years old girl. The KD patients were collected blood samples at the 5th day after the onset of fever before IVIG therapy and 24 hours after IVIG therapy and subsidence of fever. All of the KD patients underwent routine blood tests before and after therapy. And the febrile patients were collected blood samples as soon as possible after hospitalization.

Single-cell preparation and sequencing

For each patients subjected to scRNA-seq, 2 ml venous blood was collected in EDTA anticoagulant tubes and transferred to the laboratory with ice. The blood was processed within 4 hours of collection. PBMCs were isolated by density gradient centrifugation using the Ficoll-Paque medium. The cell viability should exceed 90% determined with trypan blue staining. An appropriate volume of cell suspension was calculated to contain ~12,000 cells for each sample. Single-cell capturing and library construction were performed using the Chromium Next GEM Single Cell V(D)J Reagent Kits v1.1 (10x Genomics) according to the manufacturers instructions. Briefly, the cell suspension, barcoded gel beads and partitioning oil were loaded onto the 10x Genomics Chromium Chip to generate single-cell Gel Beads-in-Emulsion (GEMs). Captured cells were lysed and the transcripts were barcoded through reverse transcription inside individual GEMs. Then cDNA along with cell barcodes were PCR-amplified. The scRNA-seq libraries were constructed by using the 5'Library Kits. Each sample was processed independently and no cell hashing was applied. The constructed libraries were sequenced on an Illumina NovaSeq platform to generate 2×

150-bp paired-end reads.

scRNA-seq data analysis

The raw sequencing data were processed by the Cell Ranger pipeline. The gene-barcode matrix of UMI counts was then analyzed with Seurat(v4.3.0)(14) for quality control, normalization, dimensional reduction, batch effect removal, clustering and visualization. For most samples, the following criteria were applied for quality control: total UMI count between 2,000 and 60,000, and mitochondrial gene percentage <5%. For the second KD patient after IVIG therapy, a lower cutoff of total UMI count between 1,000 and 40,000 was used due to its lower median UMI count per cell. The count matrix was used regularized negative binomial regression to normalize, and the top 2,000 most variable genes were identified for dimensional reduction. The samples were integrated to remove batch effects with the canonical correlation analysis (CCA), which identified cross-sample pairs of cells in a matched biological state as anchors and then used the anchors to correct technical differences between samples. The weighted nearest neighbor graph-based clustering was performed on the supervised principal component analysis (SPCA) reduced data to identify cell clusters. The cell identities were also determined with Seurat(v4.3.0)(14).

Differential expression and functional enrichment analysis

We performed differential expression analysis for each cell type on the sample level following the recommendation of Bioconductor.(15) The differential expression analysis was conducted between conditions by using Libra (v1.0.0)(16), which implements a total of 22 unique differential expression methods that can all be accessed from one function. We run Libra with pseudobulk approach, implementing the DESeq2(v1.38.3)(17) with a likelihood ratio test . We used clusterProfiler (v4.6.0)(18) for GSEA with FDR < 0.05. GSEA was performed between conditions, and the hallmark gene sets in MSigDB(v7.1)(19) were used as the database. P-values of GSEA were adjusted to FDRs and a gene set was considered significant if FDR < 0.05.

Result

Clinical symptoms and laboratory data

For the two KD patients, One was diagnosed with complete KD and one was diagnosed with incomplete KD. All patients responded to IVIG treatment and had not developed CALs. For the two febrile patients, They both had fever for 5 days with no pathogen found and negative blood cultures. In the blood routine test, we can observe that neutrophile granulocyte count and monocyte count were higher in actue KD than in febrile patients, which was decreased significantly after IVIG treatment (Table 1). Besides, C reactive protein was significant increased in acute KD.

Single-cell RNA profiling of PBMCs in KD and febrile patients

After quality control and filter, The total number of detected cells passing quality control was 36856, including 9544 cells from patients before IVIG therapy, 13569

cells after therapy, and 13743 cells from febrile patients. Based on the scRNA-seq profiles, we clustered the cells across samples with Seurat and visualized them in two-dimensional space (Figure 1). In this study, the cells were classified into 8 cell clusters including B cells, CD4+T cells, CD8+T cells, dendritic cells(DC), Monocytes, natural killer (NK) cells, other cells, other T cells; And subdivided the 8 cell clusters, B cells can be divided into naive B cells, intermediate B cells, memory B cells, Plasmablast, CD4+T can be divided into naive T cells (Tn), cytotoxic T cells (CTL), central memory T cells (TCM), effector memory T cells (TEM), and proliferating T cells. CD8+T can be subdivided into the same cell types as CD4+T, except for the absence of CTL. Monocytes can be divided into CD14 Monocytes and CD16 Monocytes.

Dynamics of immune cell types of KD and febrile patients

We compared the percentage of each immune cell type for KD patients before and after IVIG therapy, as well as febrile patients on the individual level (Figure 2), The scRNA-seq data demonstrated that KD patients after therapy had an increased percentage of CD4+T cells and CD8+T cells and a decreased percentage of monocytes compared with those before therapy. Besides, the percentage of CD8+T cell was decreased in KD patients before therapy compared to febrile patients. Moreover, we explored the change of each immune cell subtypes (Figure 3). We observed that the percentage of naive B cells decreased while the percentage of plasma cells was increased after therapy compared to KD patients before therapy. the percentage of CD16 monocytes and CD14 monocytes both decreased after therapy; for CD8+T cells and CD4+T cells, CD8 TEM and CD4 TCM were predominantly increased after therapy. Compared with febrile patients, the percentage of naive T cells was increased while the percentage of CD4 TCM and CD8 TEM was decreased in KD patients before therapy.

Differential gene expression and functions enrichment

Then we performed differential expression analysis separately for each cell types (Figure 4). We found the most DEGs (FDR < 0.05) resulted from monocytes among all PBMC populations in KD patients before and after therapy. But for acute KD patients and febrile patients, the most DEGs (FDR < 0.05) resulted from T cells. Moreover, with GSEA, we identified coordinated changes in several marker gene sets in CD4+T and CD8+T cells in KD patients before and after therapy (Figure 5), including E2F TARGETS, G2M CHECKPOINT, and MYC TARGETS V1, and the three pathways were suppressed before therapy compared to after therapy. For KD patients before therapy and febrile patients (Figure 6), B cells, CD4+T cells, CD8+T cells, and monocytes were all enriched in a marker gene set named INTERFERON ALPHA RESPONSE, and the pathway was activated in actue KD.

Discussion

According to the latest Japanese guidelines(20), the diagnostic criteria for KD, especially incomplete KD, are more complex than before. And KD has similar clinical

symptoms to some febrile diseases, which makes the early diagnosis and treatment of KD difficult, thus increasing the occurrence of CAL in children with KD. Although there are numerous diagnostic tests for KD, none of them can be applied widely. The diagnosis of KD lacks gold standard. It can reveal the immune process of KD and discover specific immune markers by scRNA-seq, so as to elucidate the pathogenesis of KD.

Our study introduced KD patients before and after IVIG therapy and febrile patients for single cell transcriptome study. We found that KD patients after therapy had an increased percentage of CD4+T cells and CD8+T cells and a decreased percentage of monocytes compared with those before therapy, which was well established by previous studies(21-23). Brogan et al.(24) have reported that Class II MHC-positive endothelial cells operate as competent superantigen-presenting cells for CD4 and CD8 lymphocytes, suggesting that activated T cells are temporarily withdrawn from peripheral circulation during acute KD. The infiltration of activated T cells expressing HLA-DR antigen in coronary vascular lesions at autopsy has been reported(25). All of the evidences showed that the decreased of T cells in PBMC in acute KD may be due to their infiltration in local tissues. CD8 TEM and CD4 TCM were predominantly increased after therapy while the percentage of CD16 monocytes and CD14 monocytes were both decreased. This is consistent with our previous findings and again demonstrates the immune cell changes in KD patients before and after therapy(21). For the comparison of acute KD with febrile patients, it was found that the percentage of CD8+ T cells decreased in acute KD. It was dominated by a decrease in the percentage of CD8 TEM and an increase of CD8 Tn. The same changes in CD8+T cell subsets could be found in the previous study(9, 21, 26), when comparing the acute KD with healthy controls. These results indicated that the effector memory cells, which can acquire rapid cytotoxic functions following activation, were the major subset contributing to the reduction of CD8+ T cells in PBMCs.

Then, by performing differential expression analysis for immune cells, we found that most DEGs resulted from T cells among all PBMC populations in acute KD compared to febrile patients, which indicated the important role of T cells in the differentiation of KD from febrile diseases. For KD patients before and after therapy, the most DEGs resulted from monocytes, which, again, validated our previous findings(21). GSEA shows that CD4+T and CD8+T cells were mainly enriched in cell cycle related pathways. Studies showed that the three pathways were related with the regulation of cell cycle and cell proliferation(27-30). Our previous study found that DEGs of B cells were enriched in cell cycle processes(21). Geng et al.(31) used scRNA-seq to compare PBMC of 2 children with KD and 2 healthy children and found that upregulated genes in monocytes of KD children were associated with cell cycle. All of these suggested that the development of KD may be related to cell cycle of immune cells and the therapeutic mechanism of IVIG may be related to cell cycle regulation. Our findings are consistent with the delayed immune development of the hygiene hypothesis(4). For acute KD and febrile patients, immune cells were found to be enriched in interferon alpha response pathway, indicating an important role of

interferon response in acute KD. Rowley et al. (32) performed deep RNA sequencing on coronary artery tissues of KD and children controls, they found that type I interferon response were significantly upregulated in KD arteritis. While Popper et al. (33) found IFN-stimulated genes were expressed at lower levels in patients with KD than in those with adenovirus infections and lower expression levels of these genes in subjects with acute KD than in both subjects recovering from KD and healthy control subjects, which suggest that an IFN response is absent in patients with KD. The role of IFN response in KD is still controversial. Our findings provide new insights into the differentiation of KD from febrile diseases and the pathogen of KD.

Study limitations

There were several shortcomings in our study. Firstly, the sample size was limited, which affected the statistical power of this study. Secondly, this study found that the development of KD may be related to the cell cycle of immune cells and the hygiene hypothesis has a strong evidence-based basis in epidemiology, but the developmental trajectory of immune cells of KD patients has not been studied and further research is needed to confirm our findings. Thirdly, This study was performed on PBMCs and might not reflect the local inflammatory responses developing in the coronary artery. At last, we only performed scRNA-seq in this study, which only reflects the transcriptome level of KD. Single-cell multi-omics sequencing allows the analysis of multiple molecular patterns for a single cell and provides better understanding of complex molecular regulatory networks. Single-cell multi-omics sequencing can be considerd to be applied in futher studies of KD.

Conclusion

In the present study, we found dynamic changes in immune cells of KD patients before and after therapy and febrile patients. GSEA revealed that T cells in the acute phase of KD were mainly enriched in cell cycle related pathways relative to after therapy, suggesting that abnormal immune cell development, proliferation and differentiation may be the potential mechanism for the immune disorder in acute KD, and the hygiene hypothesis may be the real etiology of KD. Comparing with febrile patients, we found that T cells especially CD8+T cells and the interferon response may be the key point to distinguish KD from other febrile disease.

Data availability statement

The raw sequence data are available under restricted access because of data privacy laws, and access can be obtained by reasonable request to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Shanghai Children's Hospital. Informed consent was obtained from the donors and their guardians.

Author contributions

L-jX, designed research and contributed to the final version and takes responsibility for its accuracy and integrity. GL, R-lL collected samples and performed sequencing. Q-pL, ZW analyzed data and wrote the manuscript and revised it later. The manuscript has been approved for publication by all authors. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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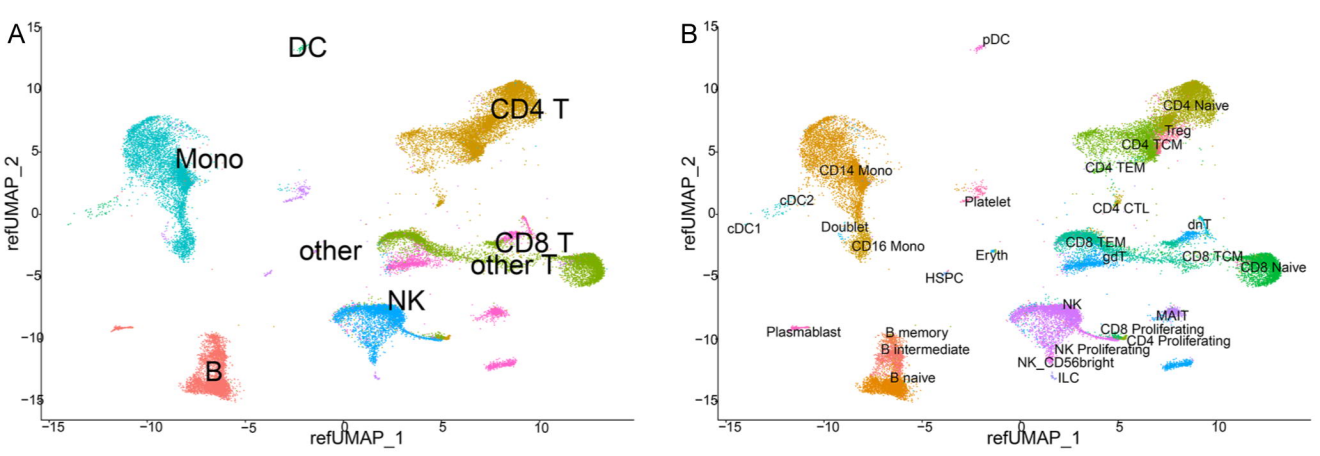
Table1. Routine blood test of KD and Fever patients .

	KD1 before	KD1 after	KD2 before	KD2 after	F1	F2
White blood cell count (10^9/L)	17.18	7.64	24.28	8.6	10.23	4.7
Neutrophil count (10^9/L)	12.95	3.58	20.23	3.98	6.9	2.04
Lymphocyte count (10^9/L)	2.47	3.04	2.6	3.76	2.36	2.2
Monocyte count (10^9/L)	1.49	0.49	1.26	0.78	0.95	0.45
Red blood cell count (10^12/L)	3.94	4.61	4.24	4.26	4.25	4.46
Platelet count (10^9/L)	244	358	462	502	237	203
C-reactive protein (mg/L)	55	11	61	22	9	7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CD4 T
Mono
other
CD8 T
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NK
B
refUMAP_2
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B
pDC
CD4 Naive
Treg
CD4 TCM
CD4 TEM
CD14 Mono
cDC2
Platelet
CD4 CTL
cDC1
Doublet
dnT
CD16 Mono
Eryth
CD8 TEM
gdT
CD8 TCM
CD8 Naive
HSPC
NK
MAIT
Plasmablast
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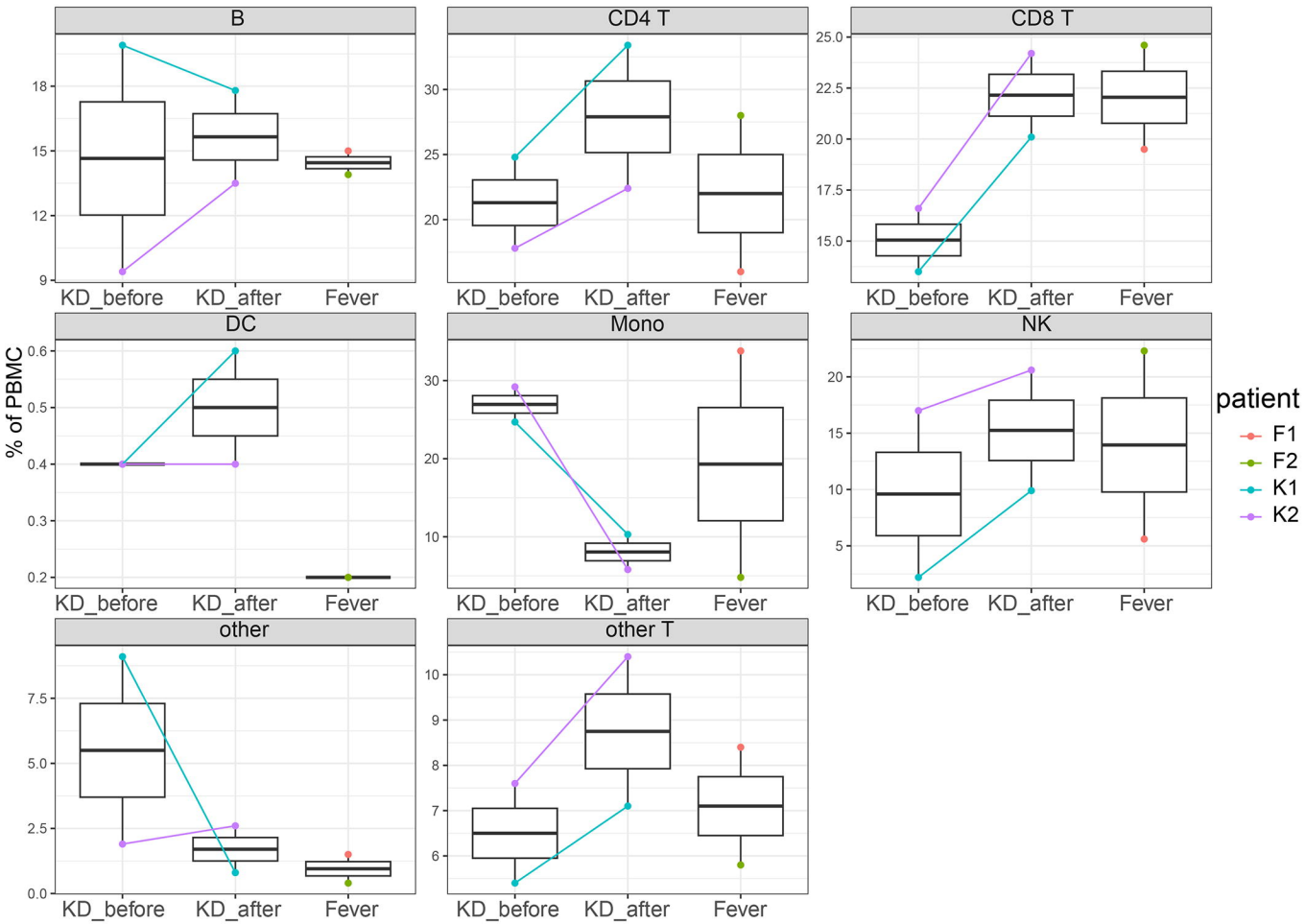
B
CD4 T
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DC
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NK
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% of PBMC
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Fever
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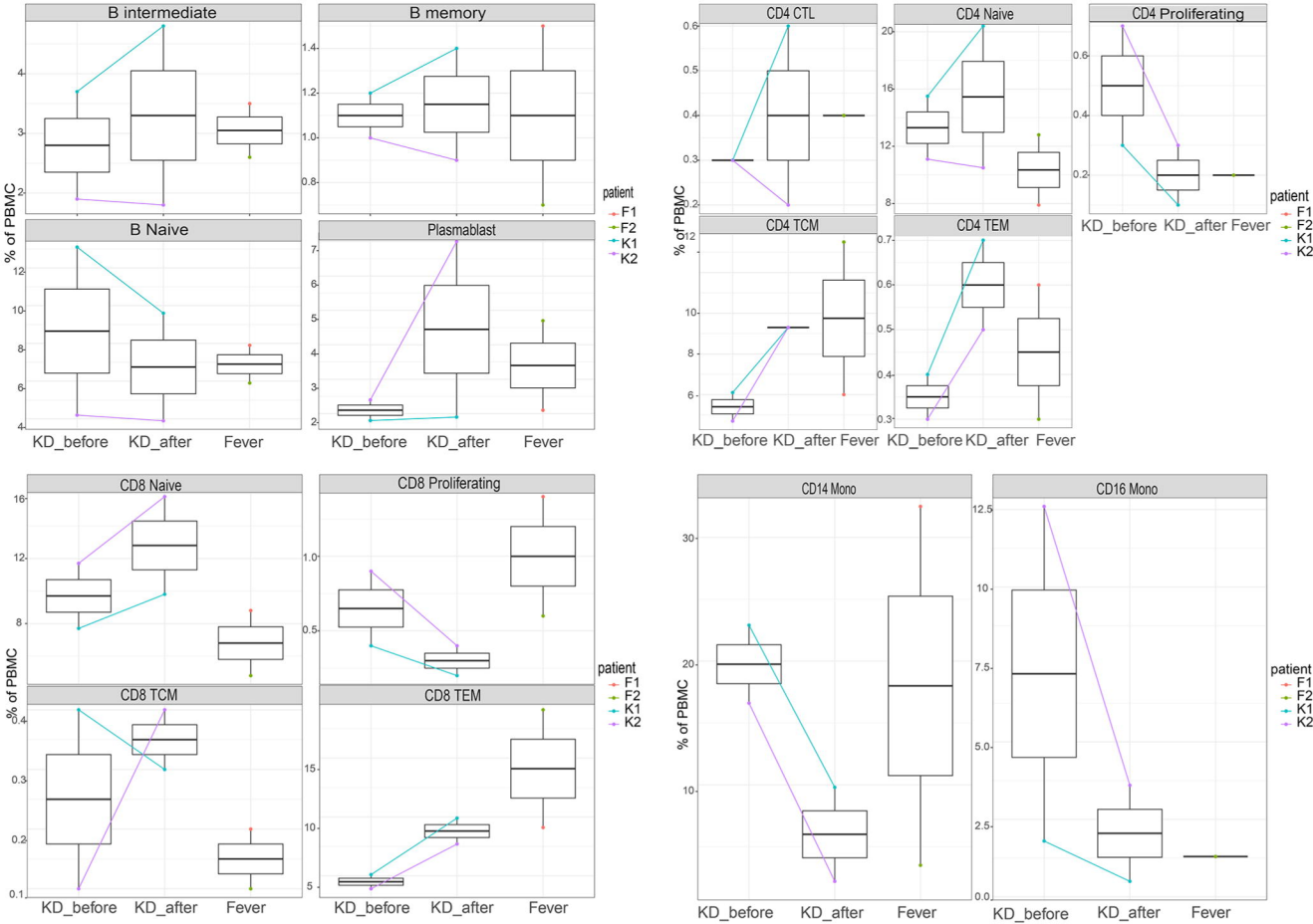
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B memory
B Naive
Plasmablast
CD4 CTL
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CD4 Proliferating
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CD8 Proliferating
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% of PBMC
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KD_after
Fever
patient
F1
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K1
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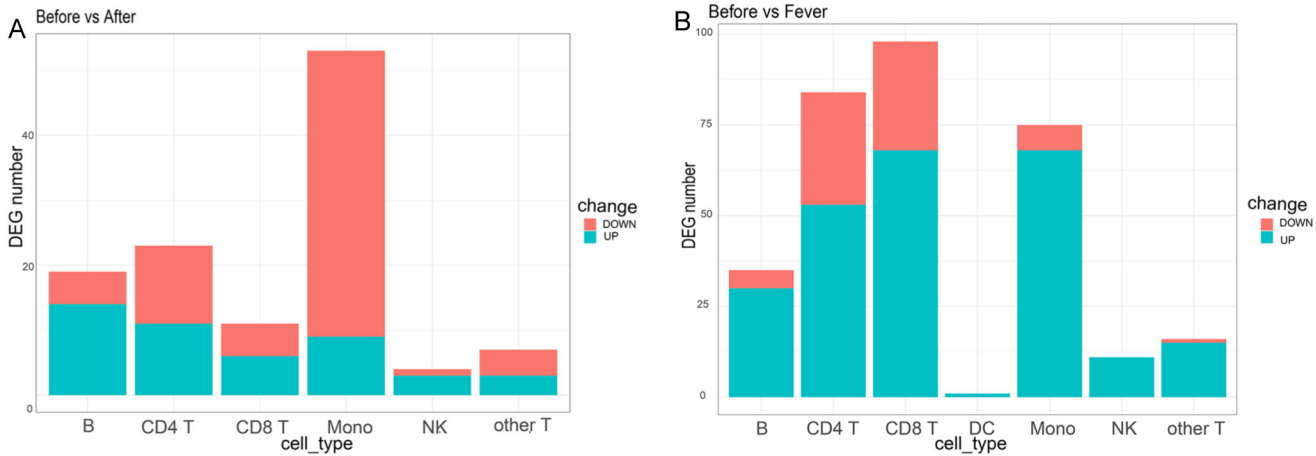
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Before vs After
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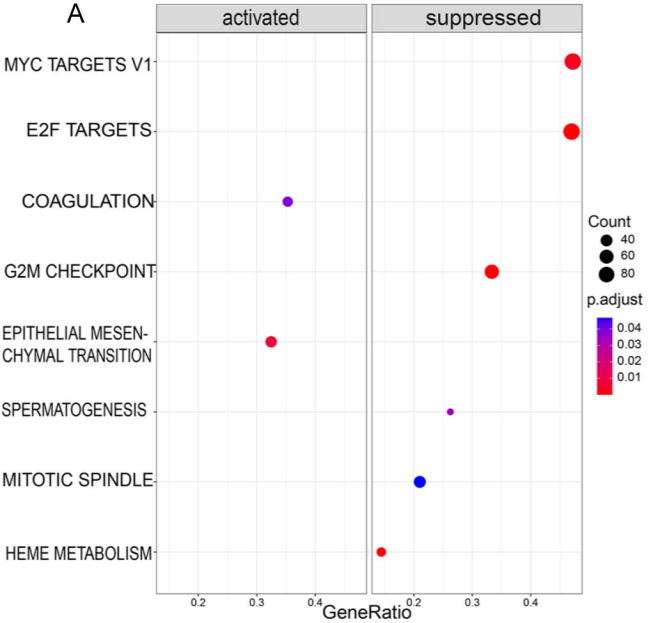
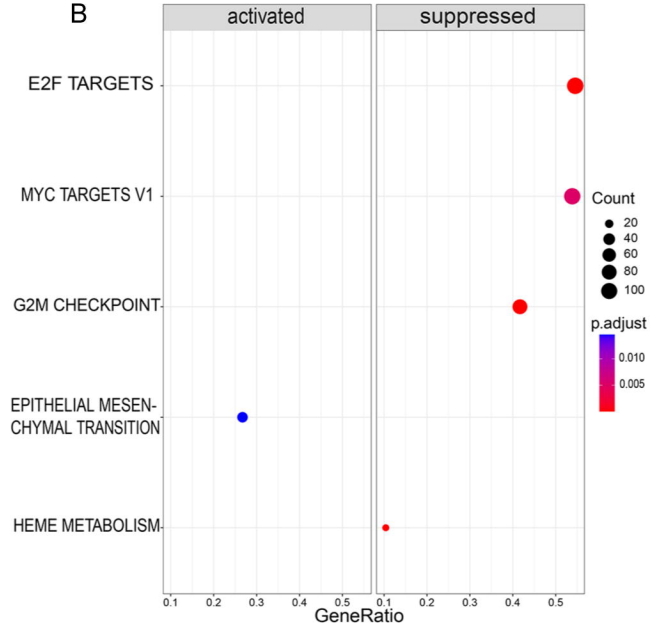
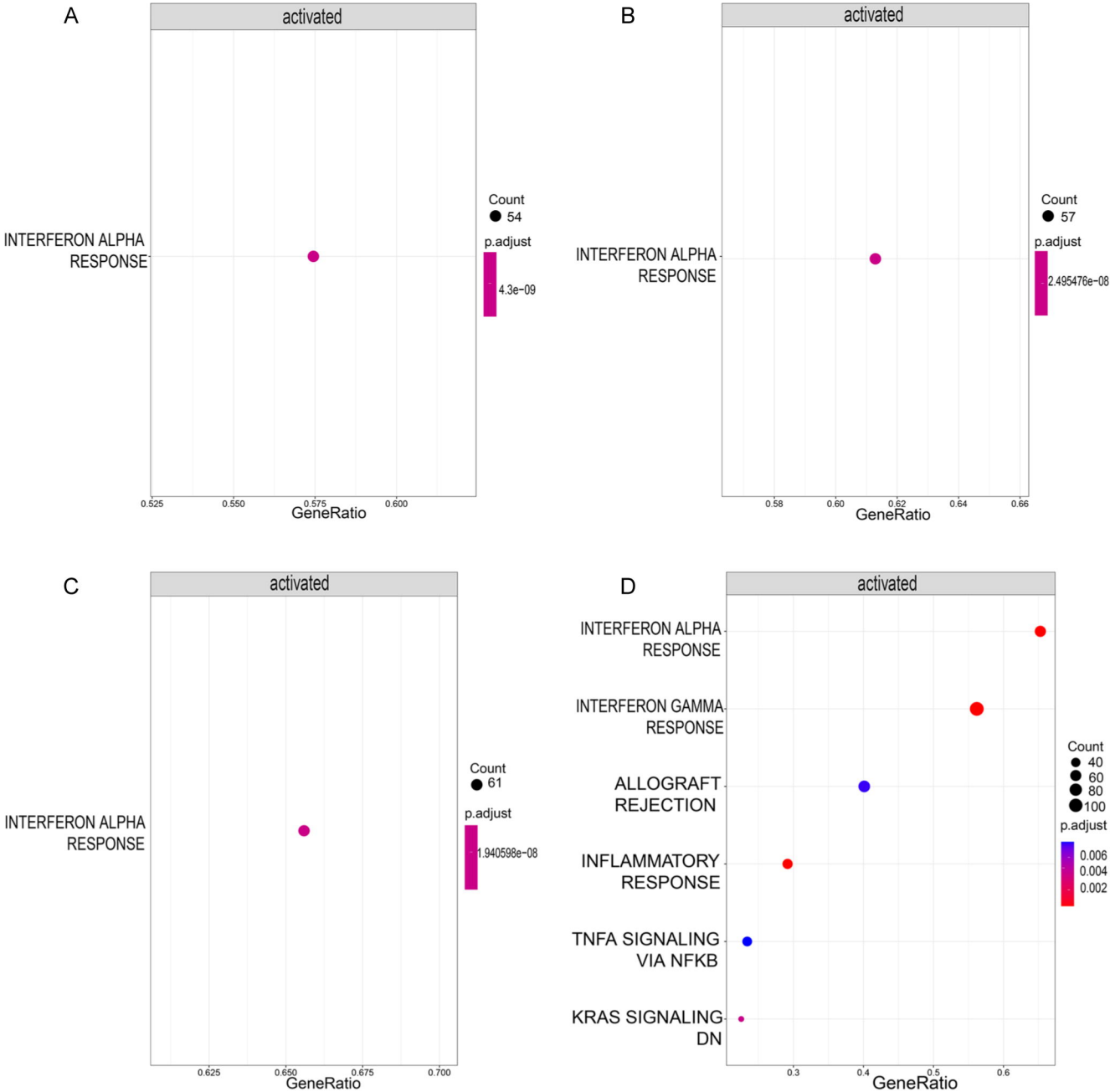
other T
cell_type
change
DOWN
UP
B
Before vs Fever
DEG number
0
25
50
75
100
B
CD4 T
CD8 T
DC
Mono
NK
other T
cell_type
change
DOWN
UP


A
activated
suppressed
MYC TARGETS V1
E2F TARGETS
COAGULATION
G2M CHECKPOINT
EPITHELIAL MESEN-
CHYMAL TRANSITION
SPERMATOGENESIS
MITOTIC SPINDLE
HEME METABOLISM
0.2
0.3
0.4
0.2
0.3
0.4
GeneRatio
Count
40
60
80
p.adjust
0.04
0.03
0.02
0.01


B
activated
suppressed
E2F TARGETS
MYC TARGETS V1
G2M CHECKPOINT
EPITHELIAL MESEN-
CHYMAL TRANSITION
HEME METABOLISM
0.1
0.2
0.3
0.4
0.5
0.1
0.2
0.3
0.4
0.5
GeneRatio
Count
20
40
60
80
100
p.adjust
0.010
0.005


A
activated
INTERFERON ALPHA RESPONSE
Count
54
p.adjust
4.3e−09
0.525
0.550
0.575
0.600
GeneRatio
B
activated
INTERFERON ALPHA RESPONSE
Count
57
p.adjust
2.495476e−08
0.58
0.60
0.62
0.64
0.66
GeneRatio
C
activated
INTERFERON ALPHA RESPONSE
Count
61
p.adjust
1.940598e−08
0.625
0.650
0.675
0.700
GeneRatio
D
activated
INTERFERON ALPHA RESPONSE
INTERFERON GAMMA RESPONSE
ALLOGRAFT REJECTION
INFLAMMATORY RESPONSE
TNFA SIGNALING VIA NFKB
KRAS SIGNALING DN
Count
40
60
80
100
p.adjust
0.006
0.004
0.002
0.3
0.4
0.5
0.6
GeneRatio