Single-cell analysis of peripheral blood mononuclear cells to reveal of cellular etiology of Kawasaki disease based on hygiene hypothesis

Qiuping Lin[1], Zhen Wang[2], Guohui Ding[3], Guang Li[4], Liqin Chen[1], Sirui Song[1], Wei Liu[1], Xunwei Jiang[1], Libing Shen[3], Tingting Xiao[1], Lijian Xie[1,5,6]

[1]Department of Cardiology, Shanghai Children's Hospital, School of medicine, Shanghai Jiao Tong University, Shanghai, China.
[2]Shanghai Institute of Nutrition and Health, University of Chinese Academy of Sciences, Chinese Academy of Sciences, Shanghai, China.
[3]Institute for Digital Health, International Human Phenome Institutes (Shanghai), Shanghai, China.
[4]Daozhi Precision Medicine Technology (Shanghai) Co., LTD.
[5]Department of Pediatrics, JinShan Hospital, Fudan University, Shanghai, China.
[6]Institute of Pediatric Infection, Immunity, and Critical Care Medicine, Shanghai Jiao Tong University School of Medicine, Shanghai, China.

Abstract

Kawasaki disease (KD) is an acute systemic vasculitis that can lead to acquired heart disease in children mostly from developed countries. Its cause is still obscure and one explanation for its emergence is called "hygiene hypothesis" stating that the immune system of a newborn must be trained by common pathogens in order to reach its full development. In this study, we analyzed peripheral blood mononuclear cells from three febrile patients and KD patients to investigate the evidence for "hygiene hypothesis" of KD. Overall single-cell expression profiles show that the biological processes of immunity, B cell activation pathway and their related entities are upregulated in febrile patients and KD patients after IVIG treatment. The differentially expressed gene analyses further demonstrate that the cell cycle genes and MYC target genes are over expressed in KD patients before treatment. Paradoxically, hematopoietic stem and progenitor cells (HSPCs) in KD patients before IVIG treatment exhibit a high expression pattern of the genes involved in immunity, immune response and B cell signaling pathway, i.e., a premature immune activation propensity. The cell differentiation trajectory is also shortened in KD patients before treatment compared with those in febrile patients and KD patients after treatment. Two cell cycle genes (HSPD1 and HSPE1) and MYC gene are early expressed in KD patients before treatment according pseudo-time analyses of cell development. The pseudo-time analyses of B cell development in three datasets indicate that a large proportion of B cells in KD patients before treatment are actually arrested in transitional state. Here, we argue that the HSPCs with a premature immune activation propensity is probably the cellular etiology behind Kawasaki disease, which lead to the symptoms of B cell dysplasia and malfunctional immune responses. Lacking of common pathogens in surroundings may be the cause of immune

hyperactive HSPCs which treat everything as pathogen. Thus, our findings in this study are an affirmative support of "hygiene hypothesis".

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. Coronary artery lesion (CAL) is a major complication of KD. High-dose intravenous immunoglobulin (IVIG) combined with aspirin is the standard treatment for KD(1). The diagnosis of KD mainly depends on clinical features. Several febrile diseases have similar clinical manifestations to KD, such as scarlet fever, EB virus infection, juvenile idiopathic arthritis, measles and adenovirus infection, leading to difficulty in diagnosis and early treatment of KD(2). The etiology of KD remains unknown. The widely proposed theories have been in the categories of environmental toxin exposure, autoimmune pathogenesis, and infectious diseases. But none of them have been proven(3). The most widely accepted etiologic hypothesis suggested that KD was caused by a kind of prevalent pathogen, which infected children with a genetic susceptibility(4). In recent years, the hygiene hypothesis, initially proposed to explain the etiology of allergic diseases, had been introduced to explain the pathogenesis of KD(5). The hygiene hypothesis assumes that lacking of early-life microbial exposure results in impaired or delayed immune maturation, leading to an abnormal response to the microbes harmless to healthy population, thus triggering KD(6, 7). The KD incidence gradually increased after its emergence in 1967(8). The epidemic data showed an important characteristic of KD that the disease is relatively rare in children less than 6 months old or greater than 5 years old, with a peak incidence in children between 6 and 24 months old in different ethnic groups (9-11). KD was followed by a prolonged period of antigen-specific 'split' T-cell anergy, which reflected a maturational defect in immune responsiveness of KD patients (12). It suggests that KD may be caused by the immune maturation delay in early childhood, which is probably due to a nearly perfect sanitary environment. The B cell development dysregulation may be the potential mechanism behind the immune maturation delay of KD(13). Immunological researches showed high IgE and low IgG levels in KD patients accompanied with a high incidence of low immunoglobulin levels (14-16). Genetic studies further showed that KD susceptibility genes are involved in B cell development and function(17-19), underscoring the crucial role of B cell immunity in KD. Though all these evidences support the hygiene hypothesis of KD, it has not been confirmed at the cellular level.

Single-cell transcriptomic sequencing (scRNA-seq) analyzes the expression profile of transcriptome at the level of individual cells, allowing the quantification of gene expression. Recent studies had employed scRNA-seq data to study the immune disorder of KD(20-22). These single cell level research revealed the dynamic changes of immune cells, differential gene expression and even biological processes in KD compared with other conditions, providing new insights into the etiology of KD and the therapeutic mechanism of IVIG.

In this study, we profiled the peripheral blood mononuclear cells (PBMCs) isolated from KD patients before and after IVIG treatment, and the PBMCs from febrile patients at single-cell resolution. We found the immunity and B cell activation pathway related biological processes were upregulated in the overall single-cell expression feature of febrile and KD patients after IVIG treatment and the activation of cell cycle related pathways was presented in most immune cells in pre-treatment KD. Analysis of peripheral blood HSPCs confirmed the premature immune activation propensity in pre-treatment KD. Single-cell trajectory analyses revealed KD patients before treatment had a shorten cell development route and an ill cell differentiation outcome. The pseudo-time analyses indicate that a large proportion of B cells in KD patients before treatment are arrested in transitional state. Our study indicates that KD occurs due to the disruption of cell cycle in immune cells, which mainly manifests as impaired B cell development and the therapeutic mechanism of IVIG for KD, possibly by rescuing B cell development. Our study illustrates the probable etiology of KD at cell level and the possible therapeutic mechanism of IVIG, providing new insights into the diagnosis and treatment of KD.

Result

Single-cell transcription profiling of PBMCs in febrile and KD patients before/after treatment

To describe the general features of single-cell expression features of febrile and KD patients before/after IVIG treatment, we collected the PBMCs from 3 febrile patients, 3 KD patients before and after IVIG treatment (Supplemental Table 1 and Table 2). After quality control and filter, the total number of detected cells passing quality control was 69300, including 19725 cells from KD patients before IVIG treatment, 26503 cells from KD patients after IVIG treatment, and 23072 cells from febrile patients. To compare their scRNA-Seq profiles, we integrated 9 samples together and clustered the cells across samples according to their expression features (Figure 1a and 1b). We identified cells to 8 major cell types including B cells, $CD4^+$T cells, $CD8^+$T cells, dendritic cells (DC), monocytes, natural killer (NK) cells, other (HSPC) cells, other T cells. The canonical gene markers were used to validate the cell clusters (Supplemental Figure 1). Figure 1c shows the percentage of major cell types s for KD patients before and after IVIG therapy, as well as febrile patients on the individual level. The scRNA-seq data demonstrated that KD patients after therapy had an increased percentage of $CD8^+$T cells($P<0.05$) and $CD4^+$T cells ($P<0.05$) and a decreased percentage of monocytes($P<0.05$) compared with those before therapy, which were well established by previous studies (22-24). Moreover, the number of B cells also shows a quite variance among febrile and KD patients before/after treatment. The separation of B cells into the detailed sub-clusters shows that febrile and KD patients after treatment have more plasmablast cells and KD patients before treatment have more B naïve and intermediate cells. The individual analysis of each patient category confirmed the result of integration analysis (Supplemental Figure 2a, 2b and 2c).

Expression features of all cells and major immune cell types in febrile and KD patients before/after treatment

We first examined the expression features of all single cells in febrile and KD patients before/after treatment. The differentially expressed genes (DEGs) were identified between KD patients before treatment and febrile and KD patients after treatment. The gene ontology (GO) analysis shows that the DEGs in febrile and KD patients after treatment share a lot of common features. They are mainly involved in the biological process of immunity and B cell activation pathways, which indicates that both immunity response and B cell development are being suppressed in KD patients before treatment (Figure 2a and 2b). Figure 2c shows the expression levels of six common genes in these two biological processes in B naive, B intermediate, plasma blast cells, natural killer cells. Their expression levels are mostly repressed in KD patients before treatment.

The differentially expressed genes (DEGs) were also identified for each major immune cell types. Gene set enrichment analysis (GSEA) revealed different major immune cell type has its own special enrichment category. For most immune cells including B cells, T cells, monocytes and NK cells, GSEA identified significantly coordinated changes of hallmark gene sets including E2F targets, G2M checkpoint, MYC targets activated in KD patients before treatment (Figure 3a). All of the pathways are cell proliferation-related gene sets. Comparing with KD patients, GSEA of all kinds of immune cells in febrile patients were enriched in interferon-alpha response and interferon gamma response (Figure 3b).

HSPCs in KD patients before treatment show a premature immune activation propensity compared with febrile and KD patients after treatment

Hematopoietic stem and progenitor cells (HSPCs) are multipotent cells that can develop to the other type of blood cells. They usually constitute a very small fraction of PBMCs and are often clustered with other cells. The integration analysis of different samples can increase the number of HSPCs and bring them together. Our integration shows that HSPCs from febrile and KD patients before/after treatment have a unique cluster of their own (Figure 1a), The number of HSPCs in febrile patients, KD patients before treatment, and KD patients after treatment is 14, 24, and 49, respectively. Although there are just a few of HSPCs in each sample dataset, it is sufficient to compare the expression difference among them with Seurat built-in function. Compared with the HSPCs from febrile and KD patients after treatment, about 120 genes are up-regulated in the HSPCs in KD patients before treatment. Their GO analysis shows that they are mainly involved in the biological processes of immunity, B cell receptor signaling pathway, innate immune response and the other immune-related functions (Figure 4a and 4b). The featured up-regulated genes in KD patients before treatment are IGHM, CD79A, CD79B, TRDC, IGHV3-11 and LIME1, which are present in both immunity and B cell receptor signaling pathway (Figure 4c). These results indicate that the HSPCs in KD patients before treatment exhibiting a premature immune activation propensity, especially early triggering B cell activation through B cell receptor signaling pathway. The HSPCs in KD patients before

treatment shows a paradoxical expression feature which is allied with the overall expression feature of febrile and KD patients after treatment.

Single-cell trajectory analyses of PBMCs in febrile and KD patients before/after treatment

Pseudotime analysis could help us reconstruct cell developmental trajectory of PBMCs in febrile and KD patients before/after treatment. Since HSPCs is the starting point of blood cell development, they are used to root the cell developmental trajectory of each sample dataset. According to pseudotime analyses, PBMCs in febrile and KD patients after treatment can be divided into five states while there are only three states of PBMCs in KD patients before treatment (Figure 5a, 5c, and 5e). It suggests that KD patients before treatment have a shorten cell development route and an ill cell differentiation outcome. The number of DEGs genes in cell developmental trajectory detected by monocle 2 package are 15364, 14819, and 15827 in febrile patients, KD patients before treatment, and KD patients after treatment, respectively. It shows that there are more genes contributing cell development in febrile and KD patients after treatment. The different cell types are plotted on the cell developmental trajectory for each sample dataset (Figure 5b, 5d, and 5f). HSPCs are set as the root for single-cell trajectory analysis in each sample dataset. They are in state 2 for febrile patients, state 3 for KD patients before treatment, and state 2 for KD patients after treatment (Supplemental Figure 3a, 3b and 3c). The single-cell trajectory analysis with the root state demonstrates that the immune cells in febrile and KD patients after treatment have a more complex development route than those in KD patients before treatment.

Expression dynamics of cell cycle genes in single-cell trajectories of febrile and KD patients before/after treatment

The developmental fate of cells is mainly governed by cell cycle genes. We have observed that the genes related to cell cycle and cell proliferation are dysregulated in KD patients before treatment. Thus, seven cell cycle genes and six cell development genes were investigated in each sample dataset. Their expression dynamics were plotted along cell developmental trajectory using HSPCs as the root state. We found that HSPD1, HSPE1, MYC, MT2A, and UBE2C genes demonstrate a different expression dynamic in three sample datasets (Figure 6a, 6b and 6c). HSPD1, HSPE1 and MYC show an elevated expression level in the early cell development stage of KD patients before treatment. The expression of MT2A and UBE2C are repressed in KD patients before treatment. Interestingly, these five genes show a different expression dynamic between febrile patients and KD patients after treatment. Compared with febrile patients, the expression of HSPD1, HSPE1 and MYC genes is repressed in KD patients after treatment. It indicates that IVIG treatment corrects the cell development dysregulation in KD patients through suppressing the expression of these three genes.

The detailed developmental path of B cells in febrile and KD patients before/after treatment

The overall profiling of PBMCs in febrile and KD patients before/after treatment shows that febrile and KD patients after treatment have the larger number of plasma blast cells than KD patients before treatment and KD patients before treatment have more B naïve and intermediate cells than the other two sample datasets. The overall expression features also demonstrated that B cell activation pathway is repressed in KD patients before treatment while the HSPCs in KD patients before treatment has an early B cell signaling pathway activation propensity. In order to elucidate the possible etiological mechanisms of KD, it is worth investigating the detailed developmental path of B cells in three sample datasets. Thus, we extracted all HSPCs and B cells from each sample dataset and used them to perform pseudotime analysis. B cells could be roughly grouped into three clusters in febrile and KD patients after treatment while they could be roughly grouped into four clusters in KD patients before treatment (Supplemental Figure 4a, 4b and 4c). The B cell developmental trajectories in febrile and KD patients after treatment display seven states while it only shows three states in KD patients before treatment (Figure 7a, 7d, and 7g). This result indicates that the developmental path of B cells is more sophisticated than that of general PBMCs. The different types of B cells were plotted along their developmental trajectories (Figure 7b, 7e, and 7h, Supplemental Figure 5a, 5b and 5c). Most B cells in KD patients before treatment were arrested in state 2 and 3. There are only some proportions of B naive cells and B intermediate and memory cells in febrile and KD patients after treatment were in state 2, 3 and 4. Most B cells in these two sample datasets could successfully enter into state 5, 6 and 7. It indicates that most B cells in KD patients before treatment were a transitional state compared with those in febrile and KD patients after treatment. CD24 is a marker gene usually expressed in transitional B cells(25, 26). Our analysis shows that CD24 is expressed in two clusters of B cells in febrile and KD patients after treatment and three clusters of B cells in KD patients before treatment. Its expression range is wider in KD patients before treatment than the other two datasets (Figure 8a, 8b and 8c). Our further analysis shows CD24 is actually a cell maker gene of the largest B cell cluster in KD patients before treatment, whose expression level is significantly higher than the other two datasets (Figure 8d). It is a direct result of the dysregulation of cell cycle in this sample dataset. The comparison of the detailed developmental trajectories of B cells in febrile and KD patients after treatment shows that B naive and plasma blast cells in these two sample datasets have different differentiation paths (Figure 7c and 7i). B naive and plasma blast cells in febrile patients were separately developed from state 2 and 6 while B naive and plasma blast cells in KD patients after treatment were directly descended from state 2. The cells of state 7 in KD patients after treatment stopped to differentiate. It shows that the rescue mechanism of IVIG for the B cell developmental path in KD patients is not the same as the normal B cell developmental path.

Discussion

KD was initially reported in 1967. Despite tremendous efforts, the etiology of KD remains unclear. Numerous etiology theories have been proposed for KD. The infection hypothesis suggests an infectious agent leads to activation of the immune system in a genetically susceptible child(27). The theory is supported by the apparent seasonality of KD, the similar clinical features with other infectious diseases and the increased levels of inflammatory markers(28-30). However, none of the potential KD infectious agents have been confirmed. A large number of studies have revealed the significant activation of innate immune response and the adaptive immune response in KD, which support the autoimmunity hypothesis. However, its low recurrence rate and the absence of a family history of autoimmune diseases in KD cases are inconsistent with the typical presentation of autoimmune disorders(1, 31, 32). In short, none of these theories of KD have been fully validated and they only partially account for the characteristics of KD. So far there is no consensus on its etiology.

Then hygiene hypothesis was introduced to explain the etiology of KD. Epidemiological and immunological data support it as a possible KD etiology and the development of B cell is a crucial factor in this hypothesis(13). In previous studies, the increase of B cell number in peripheral blood of acute KD patients has been documented. The infiltration of oligoclonal plasma cells producing IgA into the vascular wall of KD patients was also reported(33). A series of genome-wide studies identified several KD susceptibility genes are involved in B cell development and function(17-19) . These previous findings have suggested B cell immunity is crucial in the development of KD. In this study, we confirmed the impaired B cell development in acute KD, which is consistent with previous findings. We further identified such phenomenon is associated with cell cycle disorders. And the premature immune activation in peripheral blood HSPCs in acute KD suggests that B cell underdevelopment begins in bone marrow.

IVIG is very effective for treating KD. The mechanism of IVIG action on KD has not been revealed. Some people believe the anti-inflammatory role of IVIG in KD treatment while some people believe that IVIG provide passive protection against trigger antigens(34). The hygiene hypothesis proposes that the therapeutic effect of IVIG is likely due to the activation of andogenous B cell development and function. The theory well explains why the steroids, which have anti-inflammatory activity, have little therapeutic effect in KD, the recurrence rate is low in KD and the duration of fever is shorter in recurred KD. Our result showed that the therapeutic mechanism of IVIG may be by modulating the B cell cycle to a correct cell developmental clock. However, for those IVIG-resistant KD patients, who have been shown to be at a increased risk of developing CAL, the immunizing events may not occur due to unknow reasons and further research is needed.

There were several shortcomings in our study. Firstly, the sample size was limited, which affected the statistical power of this study. Secondly, the sample type is restricted. The younger samples and IVIG-resistant samples need to be collected in future studies. Thirdly, this study was performed on PBMCs and might not reflect the

local inflammatory responses developing in the coronary artery and the maturation process of hematopoietic cells in the bone marrow. Lastly, we only performed scRNA-seq in this study, which only reflected the single-cell transcriptome level of KD. Single-cell multi-omics sequencing can be considered to be applied in the further studies of KD.

Materials and methods

1. Participants and clinical characteristics

All participants were recruited from Shanghai Children's Hospital. All manipulations were approved by the Ethics Committee of Shanghai Children's Hospital (IRB number: 2022R121). Guardians of the participants had provided their informed consent in the study. KD was diagnosed according to the diagnosis criteria established by the American Heart Association(1). Two-dimensional echocardiography was used to examine whether there were coronary artery lesions during the acute and convalescent disease phases. 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. And the febrile patients were collected blood samples as soon as possible after hospitalization.

2. Single-cell RNA sequencing and data analyses

2.1 scRNA-seq library construction

Peripheral blood samples (2 mL each sample) were collected from the participants. PBMCs of participants were isolated according to standard density gradient centrifugation methods by using the Ficoll-Paque medium. The cell viability should exceed 90%. The single-cell library was constructed using the 5' Library Kits. The cell suspension was loaded onto a chromium single-cell controller (10X Genomics) to generate single-cell gel beads in the emulsion (GEMs) according to the manufacturer's protocol. Lysis and barcoded reverse transcription of polyadenylated mRNA from single cells was performed inside each GEM. Post-RT-GEMs were cleaned up, and cDNA was amplified. The barcoded sequencing libraries were generated using the Chromium Next GEM Single Cell V(D)J Reagent Kits v1.1 (10x Genomics) and were sequenced as 2×150-bp paired-end reads on an Illumina NovaSeq platform.

2.2 scRNA-seq data processing

CellRanger (Version6.0.0) software was used to process the raw FASTQ files, align the sequencing reads to the GRCh38 reference transcriptome and generate a filtered UMI expression profile for each cell. Raw gene expression matrices were read into R (Version 4.2.1) and converted to Seurat objects. The number of genes, UMI counts and percentage of mitochondrial genes were examined to identify outliers. The following criteria was applied for quality control: total UMI count between 2,000 and 60,000, and mitochondrial gene percentage <5%. After removal of low-quality cells,

the count matrix was normalized by SCTransform method, which is based on a negative binomial regression model with regularized parameters. Then all datasets from the individual samples were integrated using the “FindIntegrationAnchors” and “IntegrateData” functions in Seurat (Version 4.1.1)(35). We identified “anchors” among all individual datasets with multiple canonical correlation analysis (CCA) and used these “anchors” to create a batch-corrected expression matrix of all cells, which allowed the cells from different datasets to be integrated and analyzed. The supervised principal component analysis (SPCA) was performed to reduction and the weighted nearest neighbor(wnn) graph-based clustering was used to identify cell clusters. The cell identities were determined with multimodal reference mapping in Seurat (Version 4.1.1) (35).

2.3 Differential expression and functional enrichment analysis

DEG analysis for each cell type on the sample level following the recommendation of Bioconductor(36). The differential expression analysis was conducted between conditions by using Libra (Version 1.0.0)(37), which implements a total of 22 unique differential expression methods that can all be accessed from one function. We used “run_de” functions with pseudobulk approach, implementing the DESeq2(Version 1.36.0)(38) with a likelihood ratio test. GSEA pathway analysis was performed using clusterProfiler (Version 4.7.1)(39). Hallmark gene sets in the Molecular Signatures Database (MSigDB Version7.1)(40) served as the gene function database. P-values were adjusted to FDRs. FDRs < 0.05 was chosen as the cut-off criterion indicating a statistically significant difference.

2.4 Pseudo-time analysis of cell differentiation trajectories

Pseudo-time analysis of cell differentiation trajectories for each sample dataset was performed with R package Monocle 2(41). The expression feature and inferred cell type for each sample dataset from Seurat result was used to construct the cell dataset for Monocle analysis pipeline. We used the Monocle built-in approach named “dpFeature” to detect the variable genes that define cell’s differentiation. Its advantages are needing no prior biological knowledge and discovering important ordering genes from data itself. Dimension reduction was performed with 2 max components and "DDRTree” method. HSPCs and B cells in each sample dataset for pseudo-time analysis were extracted according to the cell identities from Seurat result.

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.

Acknowledgements

This work is jointly supported by National Natural Science Foundation of China (82170518) and the Shanghai Science and Technology Committee research Funding(22Y11909700). We would like to thank all the donors who contributed samples.

Author contributions

Z.W. and L.X. conceived and designed the project. L.X., S.S., L.C., X.J., W.L., and Q.L. collected samples and clinical information. G.D. and G.L. performed sequencing. L.S. and Q.L. performed bioinformatic analyses and discussed and interpreted the data. L.X., L.S, and Q.L. wrote the manuscript.

Competing interests

The authors declare no competing interests.

References

1. McCrindle BW, Rowley AH, Newburger JW, Burns JC, Bolger AF, Gewitz M, et al. Diagnosis, Treatment, and Long-Term Management of Kawasaki Disease: A Scientific Statement for Health Professionals From the American Heart Association. Circulation. (2017);135(17):e927-e99.doi: 10.1161/CIR.0000000000000484

2. Wright VJ, Herberg JA, Kaforou M, Shimizu C, Eleftherohorinou H, Shailes H, et al. Diagnosis of Kawasaki Disease Using a Minimal Whole-Blood Gene Expression Signature. JAMA Pediatr. (2018);172(10):e182293.doi: 10.1001/jamapediatrics.2018.2293

3. Nagata S. Causes of Kawasaki Disease-From Past to Present. Front Pediatr. (2019);7:18.doi: 10.3389/fped.2019.00018

4. Rowley AH. Kawasaki disease: novel insights into etiology and genetic susceptibility. Annu Rev Med. (2011);62:69-77.doi: 10.1146/annurev-med-042409-151944

5. Strachan DP. Hay fever, hygiene, and household size. BMJ. (1989);299(6710):1259-60.doi: 10.1136/bmj.299.6710.1259

6. Burgner D, Carter K, Webster R, Kuijpers TW. Kawasaki disease, childhood allergy and the hygiene hypothesis. Pediatr Allergy Immunol. (2011);22(7):751.doi: 10.1111/j.1399-3038.2011.01184.x

7. Lee KY, Han JW, Lee JS. Kawasaki disease may be a hyperimmune reaction of genetically susceptible children to variants of normal environmental flora. Med Hypotheses. (2007);69(3):642-51.doi: 10.1016/j.mehy.2006.12.051

8. Singh S, Vignesh P, Burgner D. The epidemiology of Kawasaki disease: a global update. Arch Dis Child. (2015);100(11):1084-8.doi: 10.1136/archdischild-2014-307536

9. Holman RC, Belay ED, Christensen KY, Folkema AM, Steiner CA, Schonberger LB. Hospitalizations for Kawasaki syndrome among children in the United States, 1997-2007. Pediatr Infect Dis J. (2010);29(6):483-8.doi: 10.1097/INF.0b013e3181cf8705

10. Nakamura Y. Kawasaki disease epidemiology and the lessons from it. Int J Rheum Dis. (2018);21(1):16-9.doi: 10.1111/1756-185X.13211

11. Holman RC, Krista Y Christensen, Ermias D Belay, Claudia A Steiner, Paul V Effler, Jill Miyamura, et al. Racial ethnic differences in the incidence of Kawasaki syndrome among children in Hawaii. Hawaii Med J. (2010);69(8):194-7.doi:

12. T W Kuijpers, A Wiegman, R A van Lier, M T Roos, P M Wertheim-van Dillen, S Pinedo, et al. Kawasaki disease: a maturational defect in immune responsiveness. J Infect Dis. (1999);180(6):1869-77.doi: 10.1086/315111

13. Lee JK. Hygiene Hypothesis as the Etiology of Kawasaki Disease: Dysregulation of Early B Cell

Development. Int J Mol Sci. (2021);22(22).doi: 10.3390/ijms222212334
14. Han JW, Oh JH, Rhim JW, Lee KY. Correlation between elevated platelet count and immunoglobulin levels in the early convalescent stage of Kawasaki disease. Medicine (Baltimore). (2017);96(29):e7583.doi: 10.1097/MD.0000000000007583
15. Makino N, Nakamura Y, Yashiro M, Ae R, Tsuboi S, Aoyama Y, et al. Descriptive epidemiology of Kawasaki disease in Japan, 2011-2012: from the results of the 22nd nationwide survey. J Epidemiol. (2015);25(3):239-45.doi: 10.2188/jea.JE20140089
16. Yamazaki-Nakashimada MA, Gamez-Gonzalez LB, Murata C, Honda T, Yasukawa K, Hamada H. IgG levels in Kawasaki disease and its association with clinical outcomes. Clin Rheumatol. (2019);38(3):749-54.doi: 10.1007/s10067-018-4339-0
17. Johnson TA, Mashimo Y, Wu JY, Yoon D, Hata A, Kubo M, et al. Association of an IGHV3-66 gene variant with Kawasaki disease. J Hum Genet. (2021);66(5):475-89.doi: 10.1038/s10038-020-00864-z
18. Lee YC, Kuo HC, Chang JS, Chang LY, Huang LM, Chen MR, et al. Two new susceptibility loci for Kawasaki disease identified through genome-wide association analysis. Nat Genet. (2012);44(5):522-5.doi: 10.1038/ng.2227
19. Onouchi Y, Ozaki K, Burns JC, Shimizu C, Terai M, Hamada H, et al. A genome-wide association study identifies three new risk loci for Kawasaki disease. Nat Genet. (2012);44(5):517-21.doi: 10.1038/ng.2220
20. Fan X, Zhou Y, Guo X, Xu M. Utilizing single-cell RNA sequencing for analyzing the characteristics of PBMC in patients with Kawasaki disease. BMC Pediatr. (2021);21(1):277.doi: 10.1186/s12887-021-02754-5
21. Geng Z, Tao Y, Zheng F, Wu L, Wang Y, Wang Y, et al. Altered Monocyte Subsets in Kawasaki Disease Revealed by Single-cell RNA-Sequencing. J Inflamm Res. (2021);14:885-96.doi: 10.2147/JIR.S293993
22. Wang Z, Xie L, Ding G, Song S, Chen L, Li G, et al. Single-cell RNA sequencing of peripheral blood mononuclear cells from acute Kawasaki disease patients. Nat Commun. (2021);12(1):5444.doi: 10.1038/s41467-021-25771-5
23. Matsubara T, Ichiyama T, Furukawa S. Immunological profile of peripheral blood lymphocytes and monocytes/macrophages in Kawasaki disease. Clin Exp Immunol. (2005);141(3):381-7.doi: 10.1111/j.1365-2249.2005.02821.x
24. S Furukawa, T Matsubara, Yabuta K. Mononuclear cell subsets and coronary artery lesions in Kawasaki disease. Arch Dis Child. (1992);67(6):706-8.doi: 10.1136/adc.67.6.706
25. Sims GP, Ettinger R, Shirota Y, Yarboro CH, Illei GG, Lipsky PE. Identification and characterization of circulating human transitional B cells. Blood. (2005);105(11):4390-8.doi: 10.1182/blood-2004-11-4284
26. Zhou Y, Zhang Y, Han J, Yang M, Zhu J, Jin T. Transitional B cells involved in autoimmunity and their impact on neuroimmunological diseases. Journal of Translational Medicine. (2020);18(1).doi: 10.1186/s12967-020-02289-w
27. Yim D, Curtis N, Cheung M, Burgner D. Update on Kawasaki disease: Epidemiology, aetiology and pathogenesis. Journal of Paediatrics and Child Health. (2013);49(9):704-8.doi: 10.1111/jpc.12172
28. Agarwal S, Agrawal DK. Kawasaki disease: etiopathogenesis and novel treatment strategies. Expert Review of Clinical Immunology. (2016);13(3):247-58.doi: 10.1080/1744666x.2017.1232165

29. Convertino M, Burns JC, Herzog L, Fabri O, Tremoulet AH, Rodó X, et al. Seasonality of Kawasaki Disease: A Global Perspective. PLoS ONE. (2013);8(9).doi: 10.1371/journal.pone.0074529
30. Rife E, Gedalia A. Kawasaki Disease: an Update. Current Rheumatology Reports. (2020);22(10).doi: 10.1007/s11926-020-00941-4
31. Hara T, Nakashima Y, Sakai Y, Nishio H, Motomura Y, Yamasaki S. Kawasaki disease: a matter of innate immunity. Clinical and Experimental Immunology. (2016);186(2):134-43.doi: 10.1111/cei.12832
32. Marrani E, Burns JC, Cimaz R. How Should We Classify Kawasaki Disease? Frontiers in Immunology. (2018);9.doi: 10.3389/fimmu.2018.02974
33. A H Rowley STS, C A Mask, L S Finn, M Terai, S C Baker, C A Galliani, K Takahashi, S Naoe, M B Kalelkar, S E Crawford. IgA plasma cell infiltration of proximal respiratory tract, pancreas, kidney, and coronary artery in acute Kawasaki disease. J Infect Dis (2000);182(4):1183–91.doi: 10.1086/315832
34. Mindy S Lo JWN. Role of intravenous immunoglobulin in the treatment of Kawasaki disease. Int J Rheum Dis. (2018);21(1):64-8.doi: 10.1111/1756-185X.13220
35. Hao Y, Hao S, Andersen-Nissen E, Mauck WM, 3rd, Zheng S, Butler A, et al. Integrated analysis of multimodal single-cell data. Cell. (2021);184(13):3573-87 e29.doi: 10.1016/j.cell.2021.04.048
36. Amezquita RA, Lun ATL, Becht E, Carey VJ, Carpp LN, Geistlinger L, et al. Orchestrating single-cell analysis with Bioconductor. Nat Methods. (2020);17(2):137-45.doi: 10.1038/s41592-019-0654-x
37. Squair JW, Gautier M, Kathe C, Anderson MA, James ND, Hutson TH, et al. Confronting false discoveries in single-cell differential expression. Nat Commun. (2021);12(1):5692.doi: 10.1038/s41467-021-25960-2
38. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. (2014);15(12):550.doi: 10.1186/s13059-014-0550-8
39. Wu T, Hu E, Xu S, Chen M, Guo P, Dai Z, et al. clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. Innovation (Camb). (2021);2(3):100141.doi: 10.1016/j.xinn.2021.100141
40. Liberzon A, Birger C, Thorvaldsdottir H, Ghandi M, Mesirov JP, Tamayo P. The Molecular Signatures Database (MSigDB) hallmark gene set collection. Cell Syst. (2015);1(6):417-25.doi: 10.1016/j.cels.2015.12.004
41. Qiu X, Mao Q, Tang Y, Wang L, Chawla R, Pliner HA, et al. Reversed graph embedding resolves complex single-cell trajectories. Nature Methods. (2017);14(10):979-82.doi: 10.1038/nmeth.4402

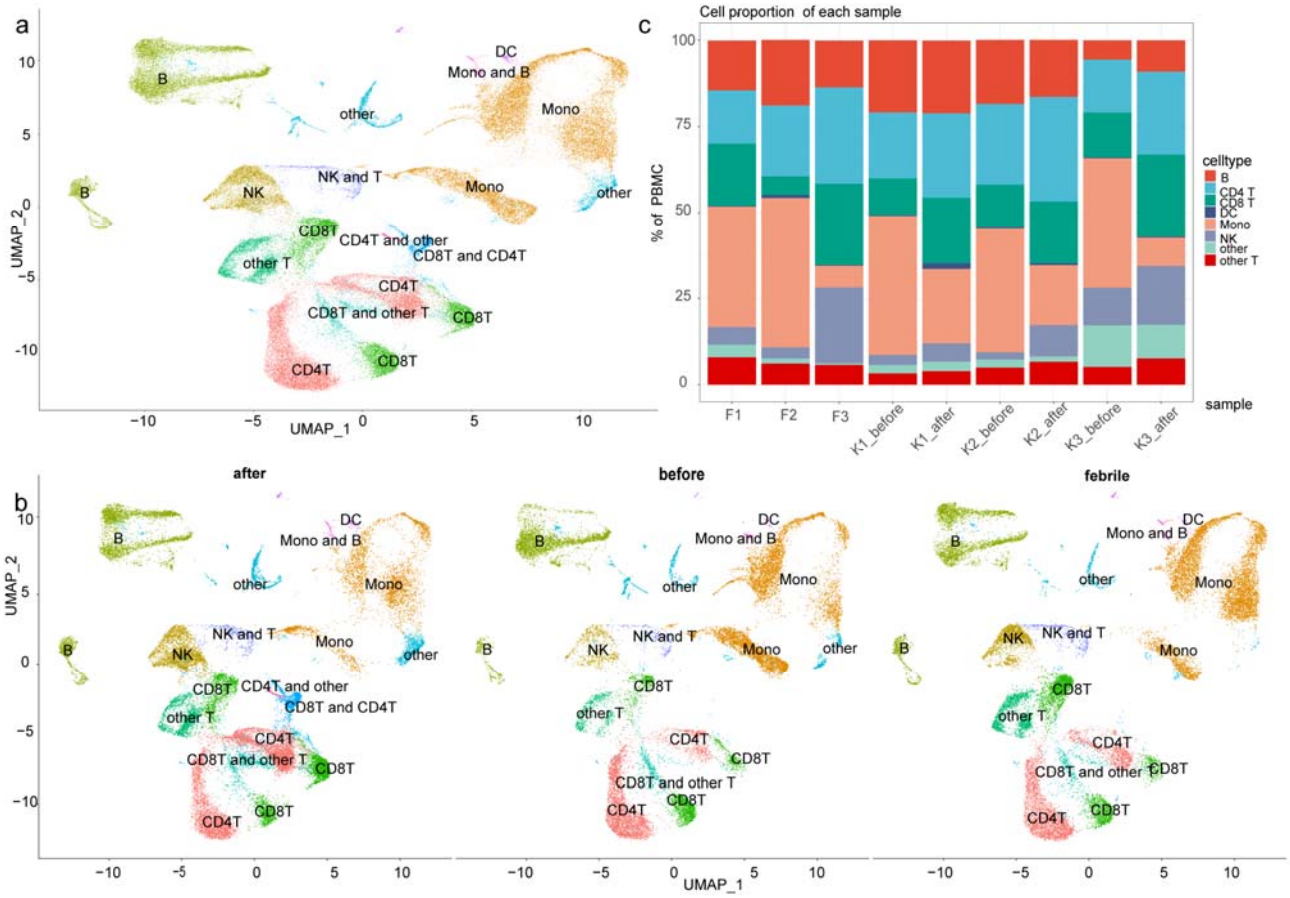

Figure 1. Single-cell profiling of PBMCs in nine samples. a. The integration single-cell profiling analysis of nine samples. b. The individual single-cell profile of febrile patients, KD patients before treatment, and KD patients after treatment. c. The proportion of different cell types in each individual sample. The inferred cell types are marked with different colors.

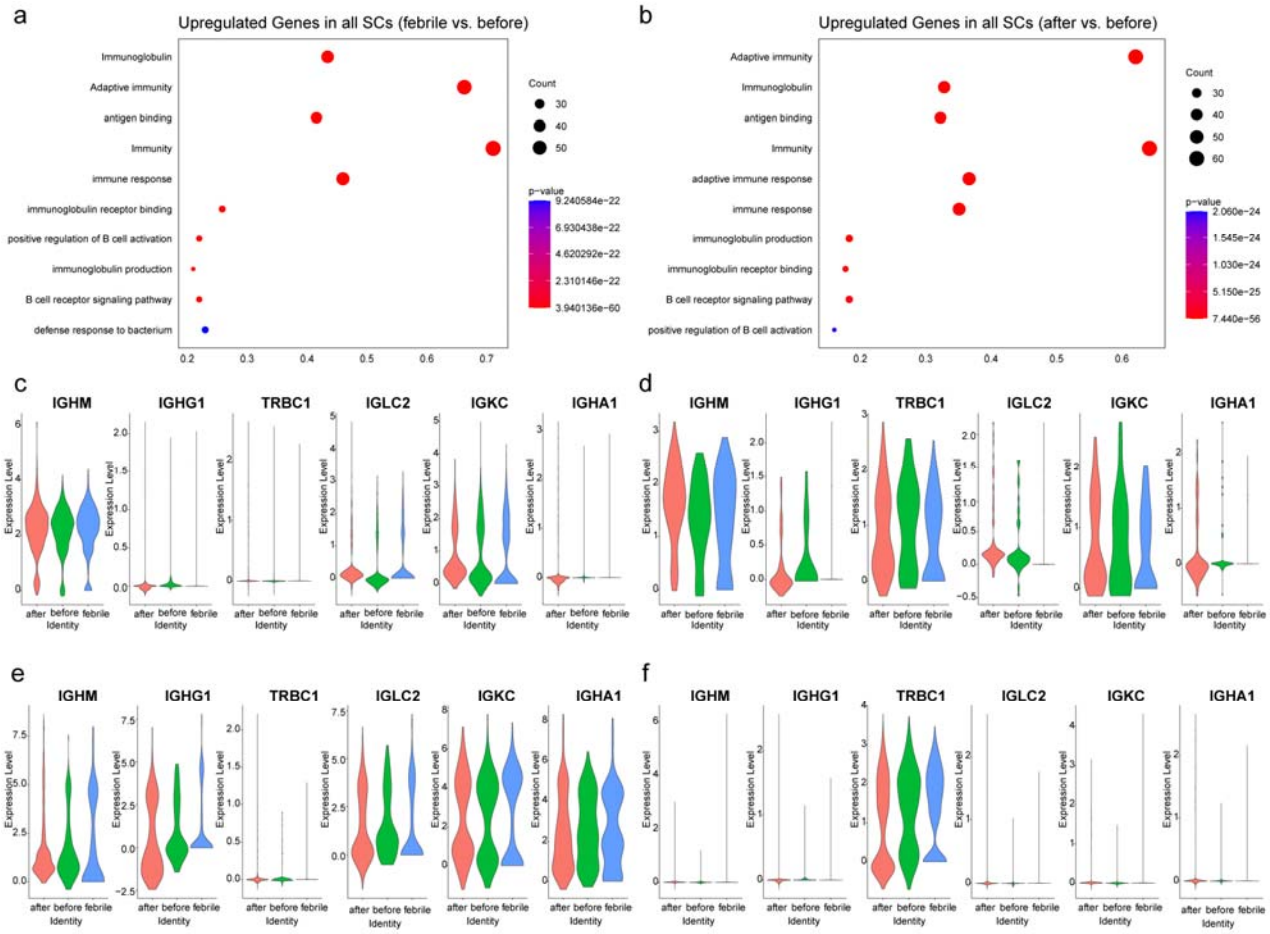

Figure 2. Analysis of DEGs in three sample datasets. a. GO term enrichment analysis of the DEGs between febrile patients and KD patients before treatment. b. GO term enrichment analysis of the DEGs between KD patients before treatment and KD patients after treatment. c. The expression level of six common genes between immunity and B cell activation pathway in B naïve cells for each sample dataset. d. The expression level of six common genes between immunity and B cell activation pathway in B intermediate cells for each sample dataset. e. The expression level of six common genes between immunity and B cell activation pathway in plasma blast cells for each sample dataset. f. The expression level of six common genes between immunity and B cell activation pathway in natural kill cells for each sample dataset.

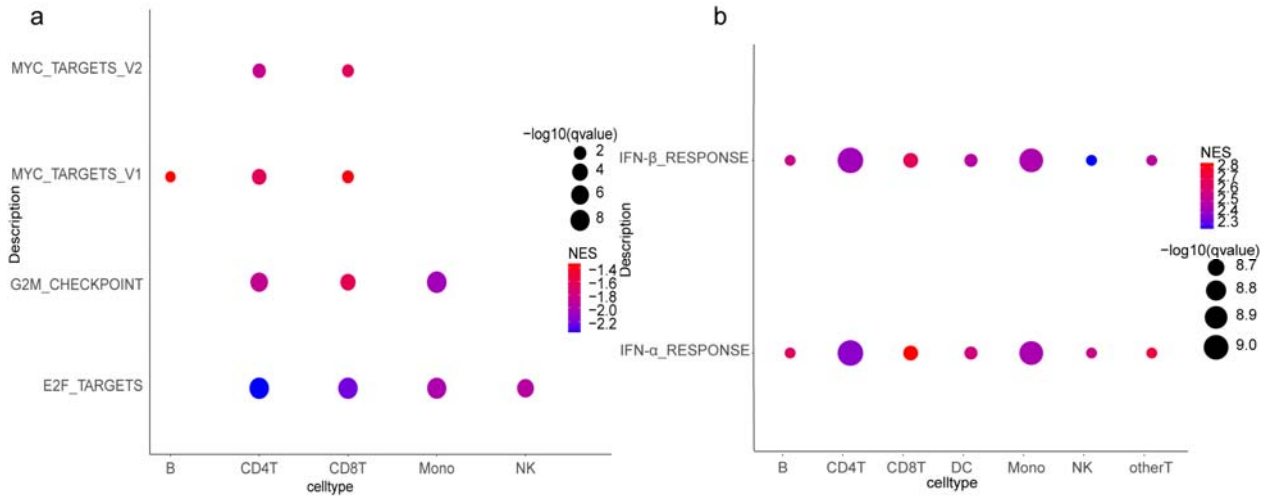

Figure 3. GSEA of the DEGs of major immune cell types in three sample datasets. a. GSEA of the DEGs between KD patients after treatment and KD patients before treatment. b. GSEA of the DEGs between febrile patients and KD patients before treatment

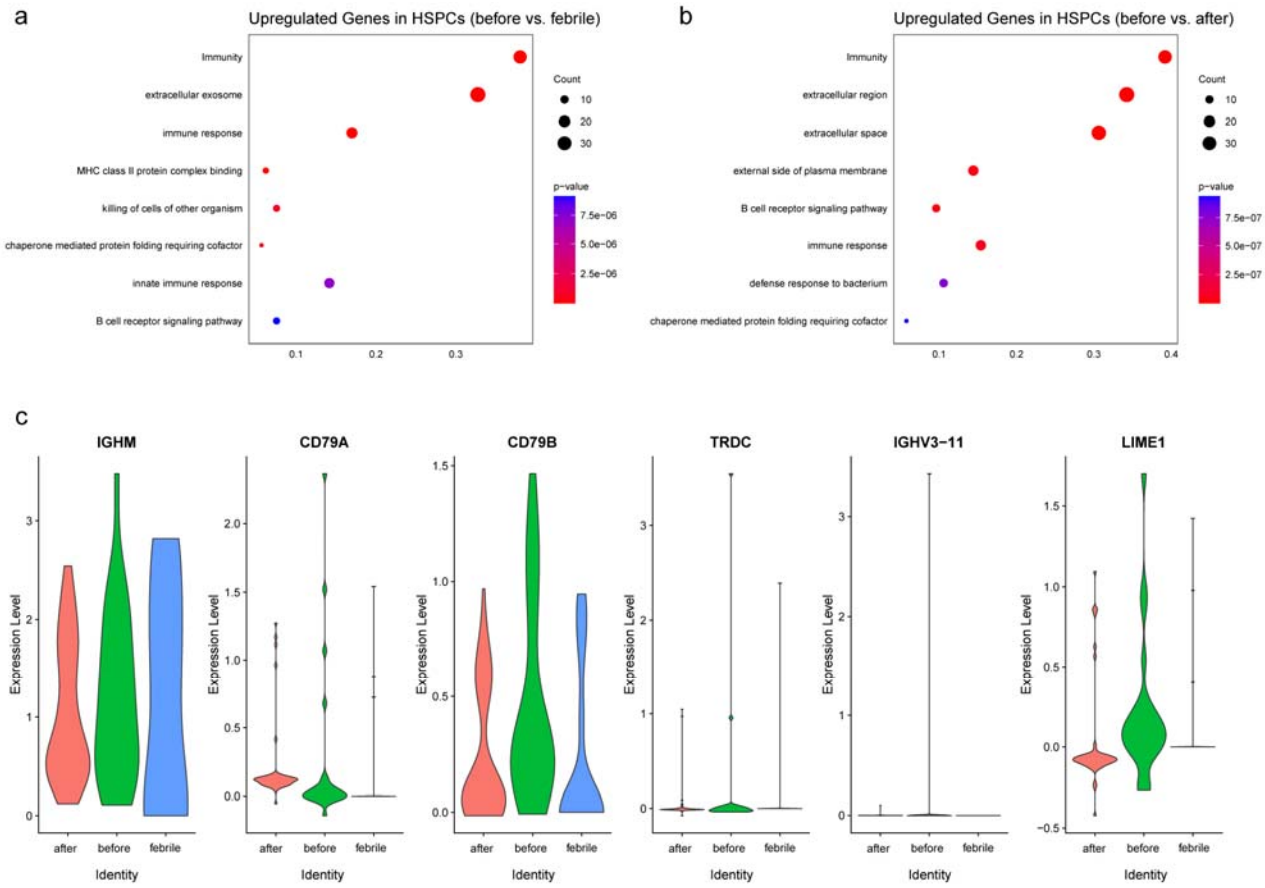

Figure 4. Analysis of the DEGs of HSPCs in three sample datasets. a. GO term enrichment analysis of the DEGs between febrile patients and KD patients before treatment. b. GO term enrichment analysis of the DEGs between KD patients before treatment and KD patients after treatment. c. The expression level of six common genes between immunity and B cell signaling pathway in HSPCs for each sample dataset.

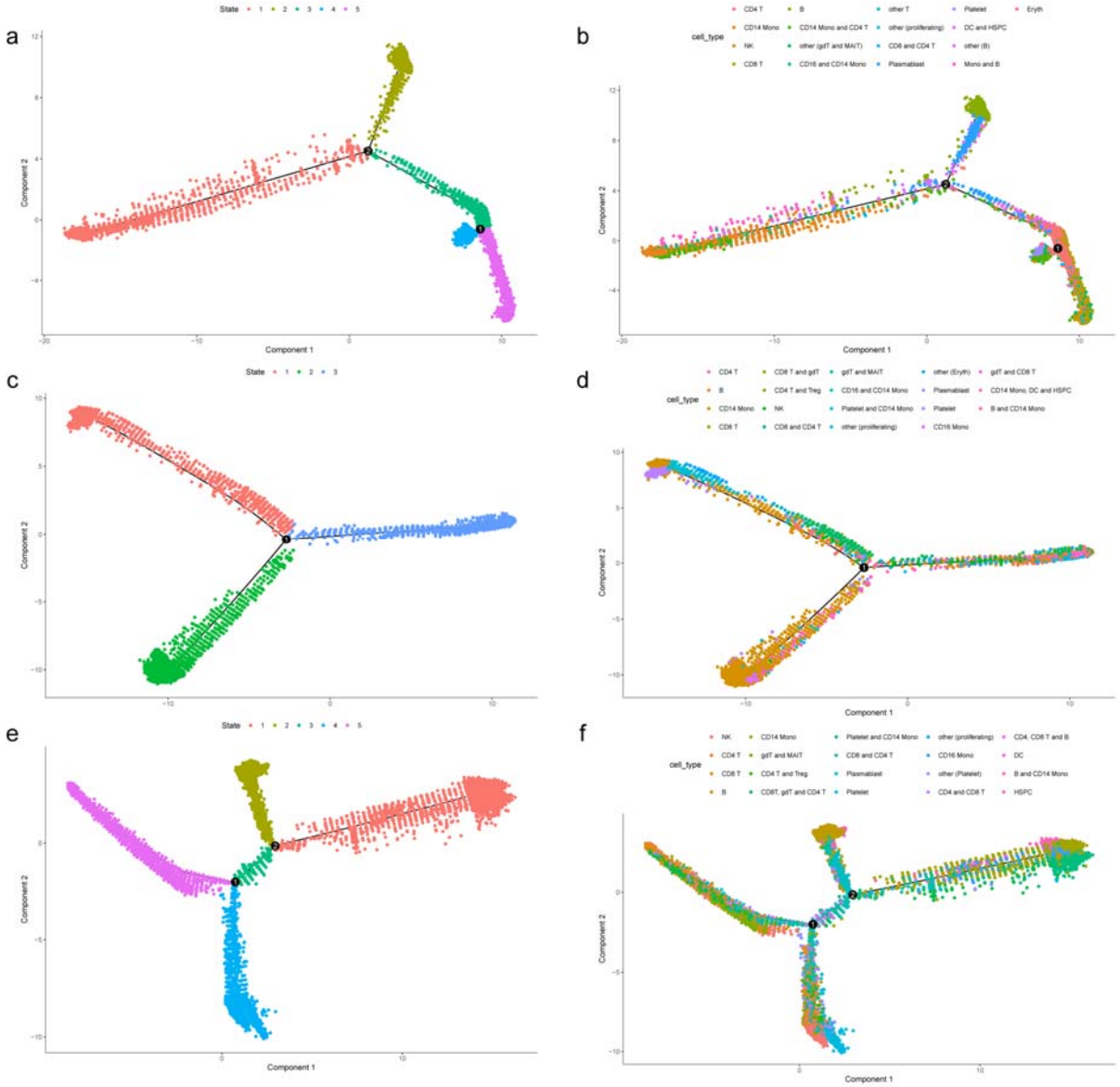

Figure 5. Pseudo-time analysis of all cells in three sample datasets. a. The differentiation trajectory of all cells in febrile patients by state. b. The differentiation trajectory of all cells in febrile patients by cell type. c. The differentiation trajectory of all cells in KD patients before treatment by state. d. The differentiation trajectory of all cells in KD patients before treatment by cell type. e. The differentiation trajectory of all cells in KD patients after treatment by state. f. The differentiation trajectory of all cells in KD patients after treatment by cell type.

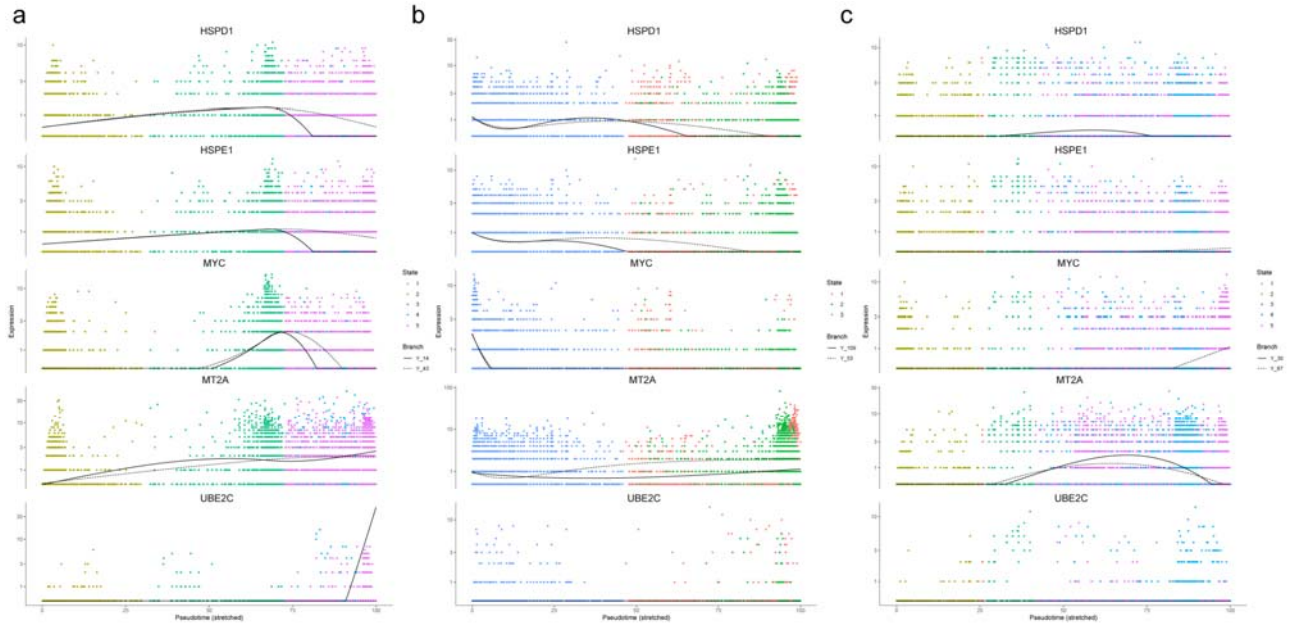

Figure 6. Pseudo-time analysis of five cell cycle and cell development genes' expression levels in three sample datasets. a. Five cell cycle and cell development genes' expression trajectories in febrile patients. b. Five cell cycle and cell development genes' expression trajectories in KD patients before treatment. c. Five cell cycle and cell development genes' expression trajectories in KD patients after treatment.

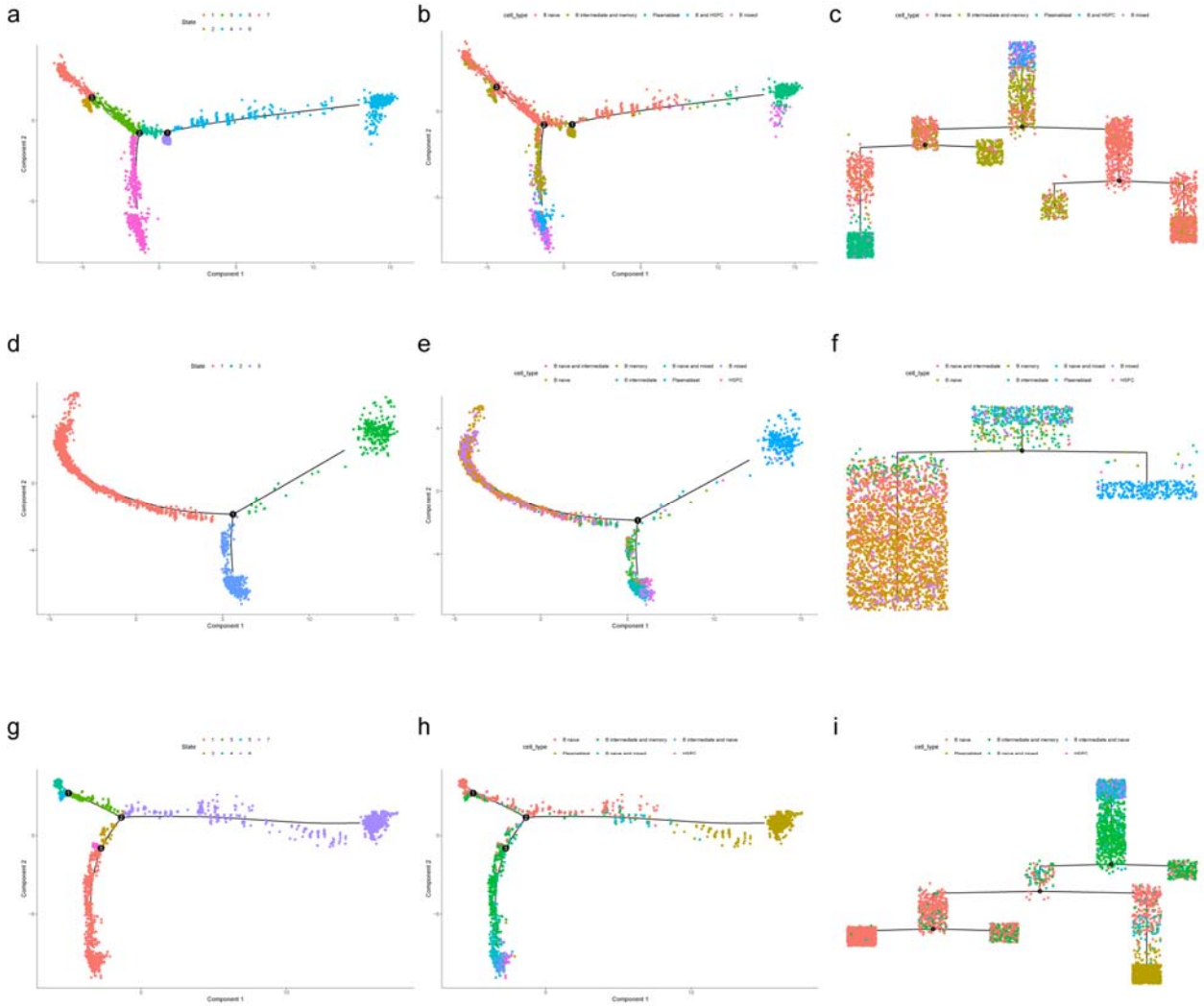

Figure 7. Pseudo-time analysis of HSPCs and B cells in three sample datasets. a. The differentiation trajectory of HSPCs and B cells in febrile patients by state. b. The differentiation trajectory of HSPCs and B cells in febrile patients by cell type. c. The differentiation trajectory of HSPCs and B cells in febrile patients by cell type displayed in tree style. d. The differentiation trajectory of HSPCs and B cells in KD patients before treatment by state. e. The differentiation trajectory of HSPCs and B cells in KD patients before treatment by cell type. f. The differentiation trajectory of HSPCs and B cells in in KD patients before treatment by cell type displayed in tree style. g. The differentiation trajectory of HSPCs and B cells in KD patients after treatment by state. h. The differentiation trajectory of HSPCs and B cells in KD patients after treatment by cell type. i. The differentiation trajectory of HSPCs and B cells in in KD patients after treatment by cell type displayed in tree style.

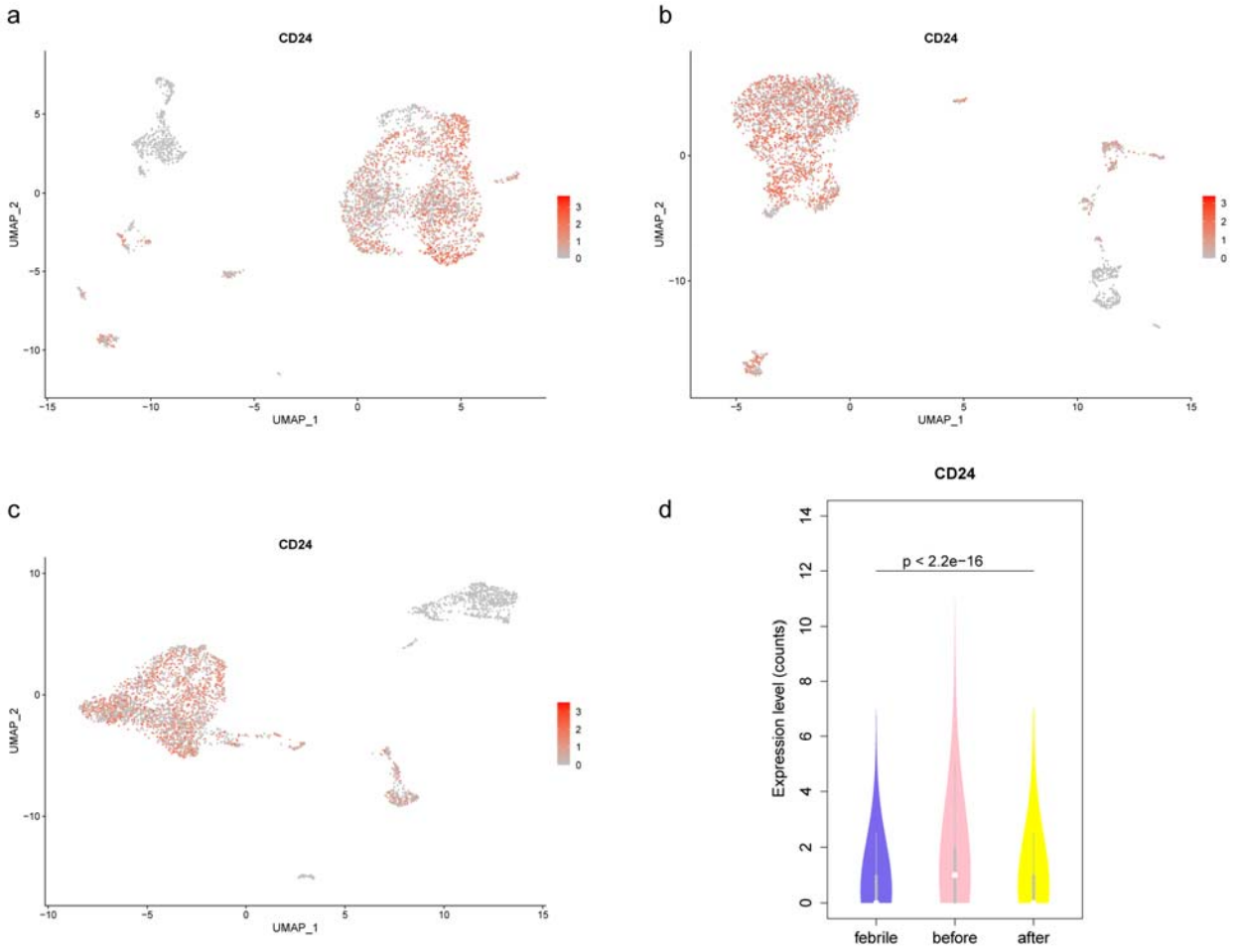

Figure 8. Analysis of CD24 expression in HSPCs and B cells in three sample datasets. a. The expression of CD24 in HSPCs and B cells in febrile patients. b. The expression of CD24 in HSPCs and B cells in KD patients before treatment. c. The expression of CD24 in HSPCs and B cells in KD patients after treatment. d. The violin plot of CD24 expression in the largest B cell cluster in three sample datasets.