Atlas of Circulating Immune Cells in Kawasaki Disease

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Abstract

Increased evidence shows that the pathogenesis of Kawasaki disease (KD) is caused by abnormal and unbalanced innate and adaptive immune responses. However, the changes in and functions of adaptive immune cells in KD peripheral blood are still controversial. In this study, three different methods, CIBERSORT, Immune Cell Abundance Identifier (ImmuCellAI), and immune cell markers, were used to evaluate the proportions and abundances of immune cells in eight KD datasets (GSE9863, GSE9864, GSE18606, GSE63881, GSE68004, GSE73461, GSE73463, and GSE64486; a total of 1,251 samples). Compared with those in normal controls and convalescent KD samples, the proportions and abundances of innate immune cells such as neutrophils, monocytes, and macrophages in acute KD peripheral blood samples were significantly increased, while those of adaptive immune cells such as B and T cells were significantly decreased. The change tendency of these immune cells was similar to that seen in other febrile illnesses but more dramatic. However, in the coronary artery tissue of KD patients, adaptive immune cells, especially B cells and CD8+ T cell subsets, were significantly increased. This result suggests that adaptive immune cells are selectively recruited from peripheral blood into the coronary arteries. In addition, we found that elevated neutrophils in peripheral blood could be used as a biomarker to assist in the differential diagnosis of KD, but we did not find immune cells that can accurately predict intravenous immunoglobulin (IVIG) response in multiple datasets.

1 Introduction

Kawasaki disease (KD) is an acute childhood vasculitis that mainly affects children from 6 months to 5 years old [1-3]. KD can cause coronary artery aneurysms (CAAs) and is the main cause of acquired heart disease in children in developed countries [4,5]. The cause is not yet clear; therefore, diagnosis and treatment are still based on experience, and KD cannot be prevented. High-dose intravenous immunoglobulin (IVIG) is the main method of treatment in the acute phase, reducing the risk of aneurysms from approximately 25% to 4%. However, approximately 20% of patients do not respond to the initial treatment of IVIG, leading to increased risk of aneurysms [6].

KD is related to the infiltration of coronary artery walls by a variety of innate and adaptive immune cells [5,7]. Based on the study of postmortem tissue in KD patients, Orenstein et al. [8] divided the vascular pathological process of KD into three steps: necrotizing arteritis develops in the first two weeks of the disease and is related to neutrophil infiltration, which gradually destroys the intima, media, and portions of the adventitia of the coronary artery; subacute/chronic vasculitis is induced by inflammatory infiltrates composed of CD8+ T cells, IgA+ plasma cells, eosinophils and macrophages; and luminal myofibroblastic proliferation (LMP), which is a process in which modified medial smooth muscle cells proliferate and gradually block the arterial lumen.

The innate immune response plays an important role in KD systemic and vascular inflammation. It is generally believed that the acute phase response of KD is mainly driven by the innate immune system [9-11]. Neutrophils, monocytes, and macrophages in the peripheral blood of acute KD are also significantly increased [12-17]. Recent studies have gradually realized that acquired immunity also plays an important

role in KD [10,18]. The decrease in the proportions and numbers of lymphocytes in the peripheral blood of KD has been well recognized [12,15,19]. However, the changes in and functions of adaptive immune cells, especially B cells and CD4+ T cell subsets in peripheral blood, are still controversial [12,13,17,20-33]. In addition, research on T cells focuses on CD4+ T cells, CD8+ T cells, Tregs, and Th17 cells, while other T cell subsets still lack relevant reports in KD.

Based on bioinformatics methods, we established a KD peripheral immune cell atlas and focused on the following two main questions.

1. How do the proportions and numbers of immune cells in peripheral blood change during acute and convalescent KD? 2. Can peripheral blood immune cells be used to diagnose KD or predict IVIG response?

The former is a basic research question that prompts exploration of the immune characteristics of KD, which will aid the understanding of the pathogenesis of KD, so that more targeted therapies can be used to replace IVIG; in contrast, the latter question prompts the identification of immune cells as biomarkers to improve timely treatment. These two questions are also the focus of current KD research.

2 Methods

2.1 Materials

Seven whole-blood RNA microarray datasets (GSE9863, GSE9864, GSE18606, GSE63881, GSE68004, GSE73461, and GSE73463) and one coronary artery high-throughput RNA sequencing dataset (GSE64486) were downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) (Table 1).

2.2 Screening of Differentially Expressed Genes (DEGs)

GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/) was used to screen the DEGs between the normal controls and KD patients (adjusted p-value <0.05). We defined common DEGs in GSE18606, GSE68004, and GSE73461 as verified differentially expressed genes (VDEGs).

2.3 Pathway and Process Enrichment Analysis

Metascape [34] (http://metascape.org/) is a web-based portal for gene function annotation analysis. The top 20 clusters with their representative enriched terms were obtained using Metascape with all VDEGs as the enrichment background. Additionally, DisGeNET [35] (https://www.disgenet.org) was used to explore VDEG-associated human diseases.

2.4 Gene Set Enrichment Analysis (GSEA)

GSEA software [36] (https://www.gsea-msigdb.org/gsea/) (version 4.0.3) was used to explore the main biological functions that caused differences between the normal controls (n=101) and KD patients (n=173). The data were derived from GSE18606, GSE68004, and GSE73461 and cleaned by the "limma" and "SVA" packages in R. "h.all.v7.1.symbols.gmt" was used as the reference gene set with 1,000 permutations for each analysis. |normalized enrichment score (NES)| [?] 1.0, p -value [?] 0.05, and false discovery rate (FDR) q-val [?] 0.25 were used as thresholds for determining statistical significance.

2.5 Calculation of the Proportions and Abundances of Immune Cells

2.5.1 Cibersort

CIBERSORT [37] is a deconvolution algorithm based on RNA-seq data. It was used to calculate the proportions of 22 immune cells in each sample, and the sum was 1. To ensure the reliability of the results, we performed 1,000 permutations and retained samples with $p< 0.05$. In this study, CIBERSORT was used to calculate the proportions of immune cells in KD samples and control samples.

2.5.2 Immune Cell Abundance Identifier (ImmuCellAI)

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ImmuCellAI [38] (http://bioinfo.life.hust.edu.cn/web/ImmuCellAI/) is a method based on single sample gene set enrichment analysis (ssGSEA) that can be used to accurately estimate the abundances of 24 immune cells from gene expression data, including 18 T cell subgroups. T cells are composed of two major groups: CD4+ T cell subsets, such as T helper cells [e.g., Th1, Th2, Th17, and T follicular helper (Tfh) cells] and regulatory T cells (e.g., iTreg, nTreg, and Tr1 cells); and CD8+ T cell subsets, such as cytotoxic T cells, exhausted T cells and mucosal-associated invariant T (MAIT) cells. In this study, ImmuCellAI was used to calculate the abundances of immune cells in KD samples and control samples.

Additionally, we also used immune cell markers to estimate the abundances of immune cells as a means of supplementary verification [39]. To facilitate visualization, each gene was normalized by $\log_2(\text{expresion}+1)$ in particular datasets.

2.6 Statistical Analysis

The Mann-Whitney U test was used for comparisons between two groups. The Kruskal–Wallis test was used for comparisons between multiple groups. The statistical analysis of the data was performed by R (version 3.6.0).$p < 0.05$ was considered to indicate statistical significance.

3 Results

3.1 The Inflammatory Response is the Most Significant Hallmark of KD

In GSE18606, GSE68004, and GSE73461, there were a total of 2,999 common DEGs between normal controls and acute KD samples, comprising 1,546 upregulated and 1,453 downregulated DEGs (Figure 1A). When these VDEGs were used as the enrichment background to conduct pathway and process enrichment analysis in Metascape, the results showed that immune-related pathways were the most frequently noted pathways. The top 20 significant enrichment clusters are shown in Figure 1B, among which "myeloid leukocyte activation", "leukocyte differentiation", "cellular response to stress", "activation of immune response", and "adaptive immune system" are the top five significant enrichment clusters. The Metascape enrichment network showed a high degree of similarity of immune-related enrichment terms within and between the top 20 clusters (Figure 1C). GSEA was also used to identify the main biological function differences between the normal controls (n=101) and acute KD samples (n=173) of GSE18606, GSE68004, and GSE73461 (Figure 1D). The results showed that the inflammatory response is the most significant hallmark of KD.
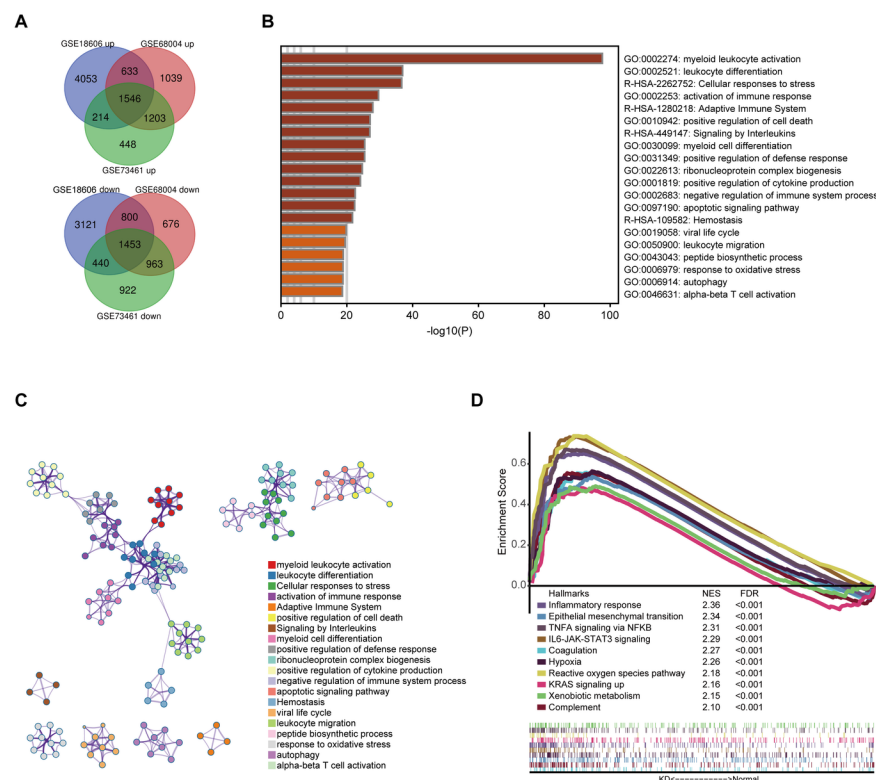
Additionally, "pneumonitis", "infection", and "bacterial infections" were the three most significant VDEG-associated human diseases identified via DisGeNET (Figure S1), which suggests that pathogens that infect through the respiratory tract could be important causes of KD.

In summary, the immune response is the most important feature of KD.

3.2 In Acute KD Peripheral Blood, Innate Immune Cells are Increased, While Adaptive Immune Cells are Decreased

To describe the dynamic changes in peripheral blood immune cells in KD, CIBERSORT was used to calculate the proportion of each of the 22 immune cell types based on KD microarray data. We separately determined the composition of peripheral blood immune cells in the normal control group (n=101, GSE18606, GSE68004, and GSE73461), acute KD group (n=480, GSE18606, GSE63881, GSE68004, GSE73461, and GSE73463) and convalescent KD group (n=101, GSE18606, GSE63881, and GSE73463) (Figure 2A-C). We found that the proportion of innate immune cells represented by neutrophils, monocytes, and macrophages was significantly increased and that of adaptive immune cells represented by B cells and T cell subsets was significantly decreased in acute KD (Figure 2D). In convalescent KD, the aforementioned changes in the proportion of immune cells were restored.

We determined the differences in immune cell proportions between normal and acute KD samples, and between acute KD and convalescent KD samples in GSE18606, GSE63881, GSE68004, GSE73461, and GSE73463 (Figure 2E and Figures S2 and S3). The results that show the same significant trend in the comparison of 4 or more datasets are considered to have high credibility. Using this as a standard, our results showed that compared with the respective values in normal and convalescent KD samples, the proportions of

monocytes, M0 macrophages, and neutrophils in acute KD samples were significantly increased, while those of memory B cells, CD8+ T cells, resting CD4+ T cells, resting natural killer (NK) cells, activated NK cells, and M2 macrophages were significantly decreased.
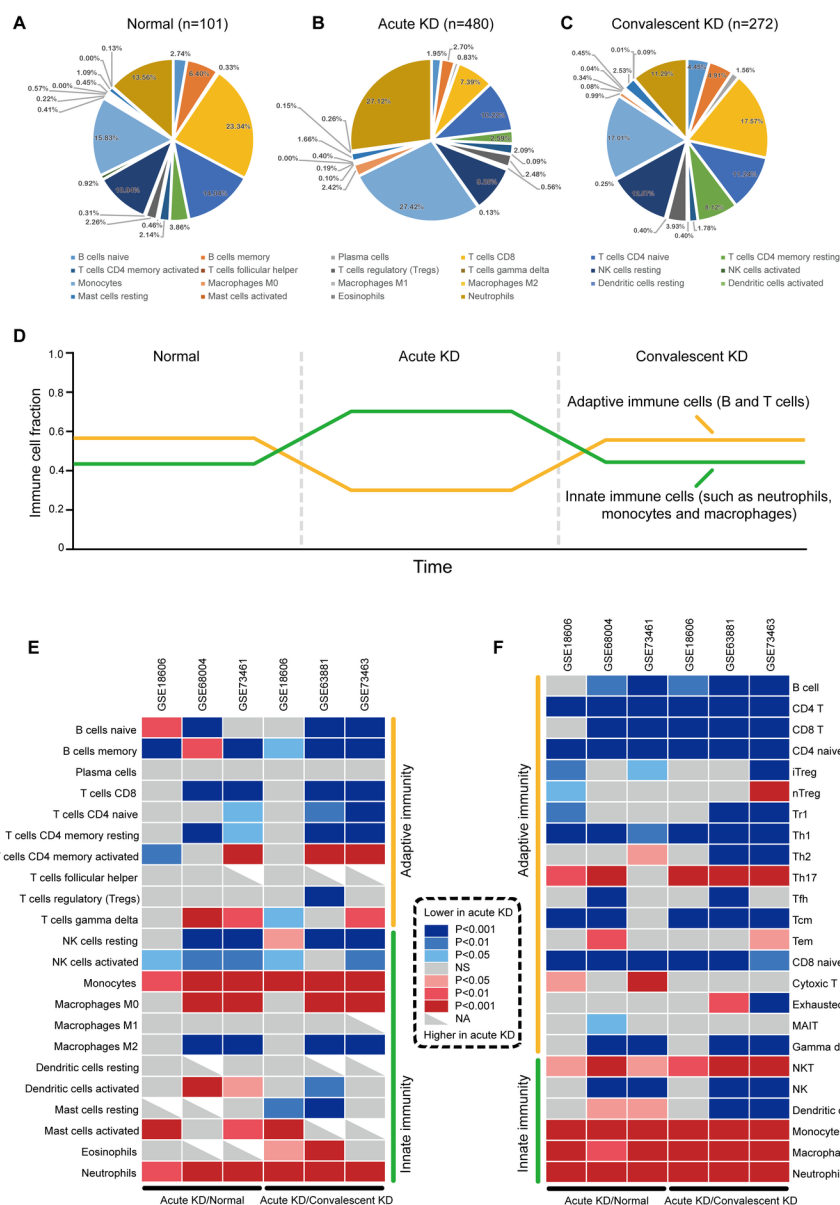
Similarly, we determined the differences in immune cell abundances (ImmuCellAI) (Figure 2F, Figures S4 and S5, and Tables S1 and S2). The results showed that compared to the respective values in normal and convalescent KD samples, the abundances of Th17 cells, natural killer T (NKT) cells, neutrophils, monocytes, and macrophages in acute KD samples were significantly increased, while those in B cells, CD4+ T cells, CD8+ T cells, CD4+ naive T cells, Th1 cells, central memory T (Tcm) cells, naive CD8+ T cells, gamma delta T ($\gamma\delta$ T) cells and NK cells were significantly decreased.

In addition, comparison of immune cell markers in GSE9863, GSE18606, GSE63881, GSE68004, GSE73461,

and GSE73463 (Figures S6-S8) also showed that CD68 (a macrophage marker) and CXCR2 (a neutrophil marker) levels in acute KD samples were significantly higher than those in normal and convalescent KD samples, while MS4A1 (a B cell marker), CD8A (a cytotoxic T cell marker), CD4 (a T helper cell marker), CXCR5 (a Tfh marker), B3GAT1 (an NK cell marker) levels were significantly decreased.

In summary, compared with the respective values in normal controls and convalescent KD samples, the proportions and abundances of innate immune cells represented by neutrophils, monocytes, and macrophages were significantly increased while those of adaptive immune cells represented by B cells and T cell subsets were significantly decreased in acute KD peripheral blood samples.

3.3 The Changes in Peripheral Blood Immune Cells in Acute KD are Similar to those Derived from Febrile Controls but More Dramatic

To explore the differences between peripheral blood immune cells in KD and other febrile diseases, the febrile controls of GSE68004 and GSE73461 were also included in the study. The results showed that compared with those in the normal controls, the immune cell changes in the acute KD samples and febrile controls were similar. Both showed increased innate immune cells and decreased adaptive immune cells, but the degree of change in the acute KD samples was more dramatic (Table 2). In particular, the decrease in CD4+ T cells and CD8+ T cells and the increase in neutrophils in KD peripheral blood samples were specific compared to febrile controls (Table 2 and Figure S9). Peripheral blood neutrophil abundance performed well in the differential diagnosis of KD samples from normal controls and high fever controls (Figure S10). In addition, the InfiltrationScore, which represents the degree of immune response in ImmuCellAI, was also highest in acute KD (Table 2).

3.4 There are no KD Peripheral Blood Immune Cells that can Accurately Predict the IVIG Response

Generally, a patient with persistent or recurrent fever [?]36 h after the completion of the initial IVIG infusion is defined as an IVIG nonresponder [40]. In GSE63881, IVIG nonresponders accounted for a minority of the KD patients (61/171), but they accounted for the majority of the patients with a diagnosis of coronary angioma (15/20) (Figure 3A). Therefore, we explored whether peripheral blood immune cells can be used to predict the IVIG response. We compared the proportions and abundances of immune cells between IVIG responders and IVIG nonresponders in acute KD and convalescent KD (Figure 3B-E and Figures S11-S13). However, no peripheral blood immune cells that could accurately predict the IVIG response in multiple datasets were identified.
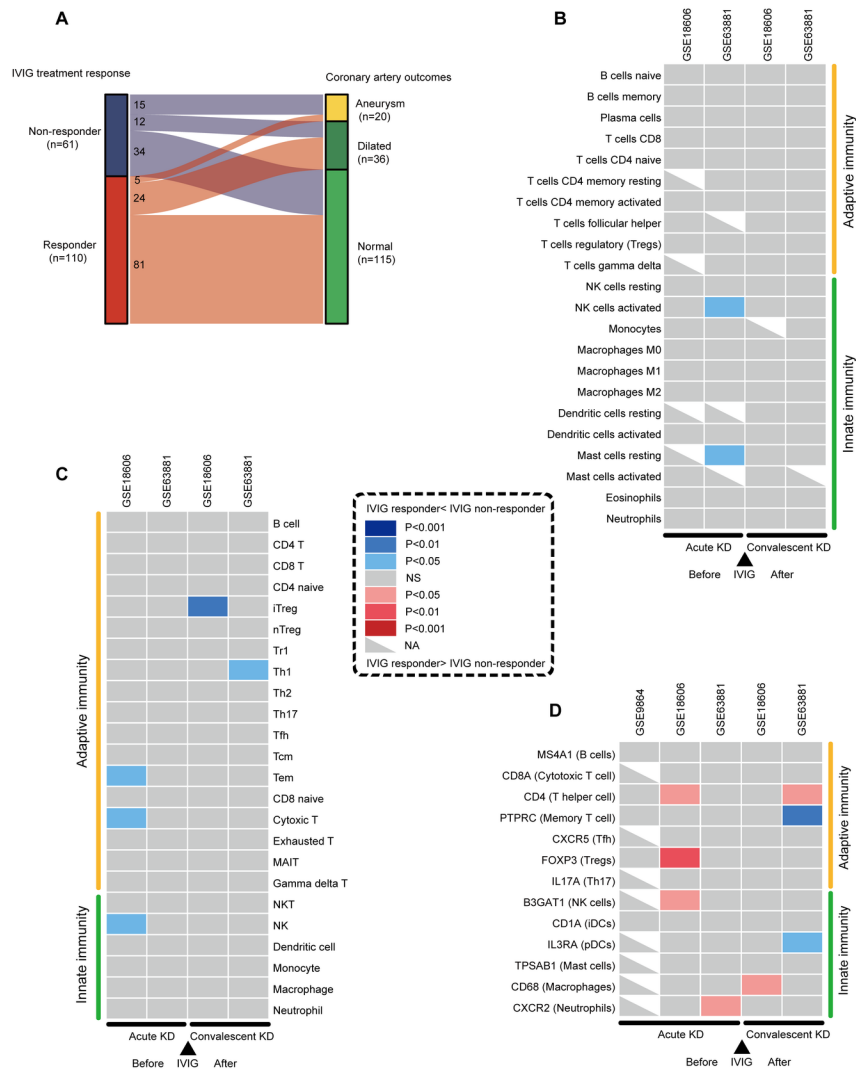
In addition, we also analyzed the relationship between KD peripheral blood immune cells and coronary aneurysm formation in GSE63881, and we did not find any immune cells that were highly related to aneurysm formation (Figure S14).

3.5 There is High Infiltration of Adaptive Immune Cells in KD Coronary Arteries
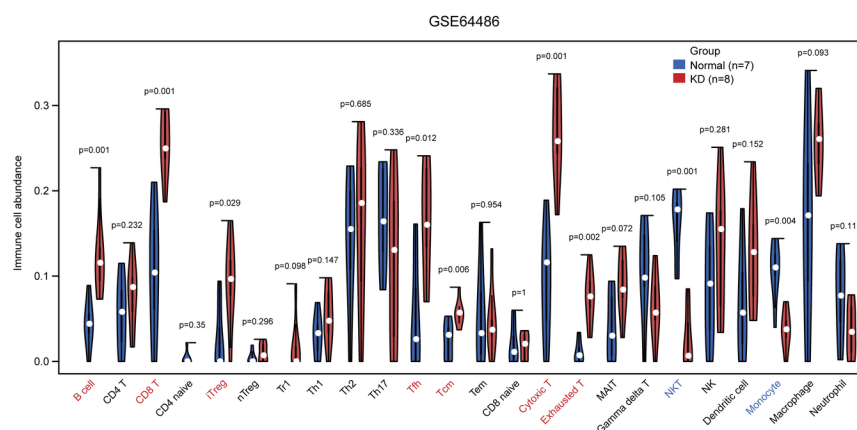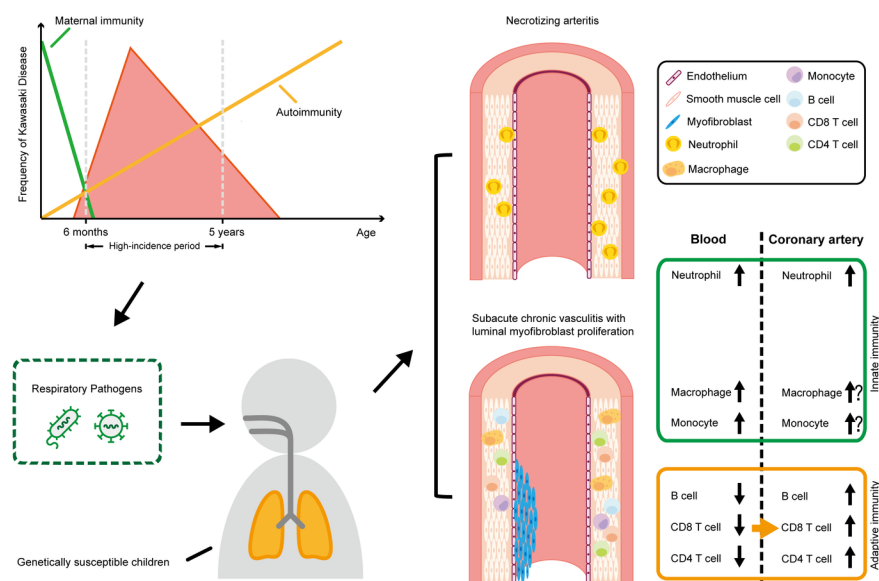
We further compared the difference in immune cell infiltration between KD coronary arteries and normal coronary arteries in GSE64486. The ImmuCellAI results showed that in KD coronary arteries, the abundances of B, CD8+ T, iTreg, Tfh, Tcm, cytotoxic T and exhausted T cells were significantly increased, while those of NKT cells and monocytes were significantly decreased (Figure 4). In addition, the results of immune cell biomarkers showed that almost all the markers of immune cells showed an upward trend, including MS4A1 (B cells), CD8A (cytotoxic T cells), CD4 (T helper cells), PTPRC (memory T cells), and CXCR2 (neutrophils), which reached a statistically significant level (Figure S15).

In summary, adaptive immune cells, especially B cells and CD8+ T cell subsets, are significantly increased in KD coronary arteries.

4 Discussion

In this study, by using three different algorithms to evaluate the proportions and abundances of immune cells in a total of 1,251 samples from eight GEO datasets, we established an atlas describing the changes in KD peripheral blood immune cells. The results show that compared with those in normal controls and convalescent KD samples, the proportions and abundances of innate immune cells such as neutrophils, monocytes, and macrophages in acute KD peripheral blood samples are significantly increased, while those of adaptive immune cells such as B and T cells are significantly reduced. The change tendency of these immune cells is similar to that seen in other febrile illnesses but more dramatic. In the coronary arteries of KD, adaptive immune cells, especially B cells and CD8+ T cell subsets, are significantly increased. This finding suggests that adaptive immune cells are selectively recruited from peripheral blood into the coronary arteries. In addition, we found that elevated neutrophils in peripheral blood could be used as a biomarker to assist in the differential diagnosis of KD, but we did not find immune cells that could accurately predict IVIG response in multiple datasets.

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Maternal immunity

Frequency of Kawasaki Disease

Autoimmunity

6 months 5 years Age

High-incidence period

Necrotizing arteritis

Respiratory Pathogens

Genetically susceptible children

Subacute chronic vasculitis with luminal myofibroblast proliferation

Endothelium Monocyte
Smooth muscle cell B cell
Myofibroblast CD8 T cell
Neutrophil CD4 T cell
Macrophage

Blood Coronary artery

Neutrophil ↑ Neutrophil ↑
Macrophage ↑ Macrophage ↑?
Monocyte ↑ Monocyte ↑?

Innate immunity

B cell ↓ B cell ↑
CD8 T cell ↓ CD8 T cell ↑
CD4 T cell ↓ CD4 T cell ↑

Adaptive immunity

KD pathogenesis is currently understood to include a pathologically amplified immune response in children who are genetically susceptible to pathogens that are believed to be widely dispersed in the environment and to enter through the respiratory tract [2,41,42] (Figure 5). Our results show that "pneumonitis", "infection", and "bacterial infections" are the three most significant VDEG-associated human diseases according to DisGeNET, which supports that respiratory tract infection is an important cause of KD. In addition, our research shows that the change trend of peripheral blood immune cells in acute KD is similar to that seen in febrile illnesses caused by bacterial or viral infection but is more dramatic, which also suggests that KD is an excessive immune response to common infectious agents. In addition, outbreaks of KD around the world during the COVID-19 pandemic have shown that the agents that induce KD are nonspecific [43,44].

Increased evidence suggests that KD is caused by abnormal and imbalanced innate and adaptive immune responses [18,45]. Our functional and pathway enrichment analysis results also showed that the inflammatory response is the most significant hallmark of KD. At present, many studies have described the changes in KD peripheral blood immune cells (Table 3). The significant increase in innate immune cells in acute KD has been well recognized. However, the changes in and functions of adaptive immune cells, especially B cells and CD4+ T cell subsets, are still controversial. In recent years, research has suggested that B cells in the peripheral blood of acute KD are elevated [12,16,17,20-23,46-53]. However, our results show that B cells decrease significantly in acute KD and return to the levels in normal samples in convalescent KD. In addition, it is worth mentioning that our study is the first to use ImmuCellAI to calculate the abundances of 18 T cell subsets in KD peripheral blood, and the abundances of most T cell subsets have not been reported in KD. Our results showed that compared with the respective values in normal controls and convalescent KD samples, the abundances of CD4+ T cells, CD8+ T cells, CD4+ naive T cells, Th1 cells, Tcm cells, naive CD8+ T cells, and γδ T cells in acute KD samples were decreased significantly, while the abundances of Th17 cells and NKT cells were significantly increased. The detailed classification of T cells helps us to understand the features and functions of T cell subsets in KD more accurately.

KD is closely related to the infiltration of coronary artery walls by a broad variety of innate and adaptive immune cells [5,7,9,72] Research on postmortem tissues of KD patients has indicated that neutrophils are the first responders to invade the arterial wall, followed by monocytes/macrophages, dendritic cells and CD8+ T cells [7,8]. These infiltrating immune cells release proinflammatory cytokines such as IL-1β and

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TNF to promote the development of vascular endothelial cell injury and CAA [7]. We also observed an increase in the abundances of adaptive immune cells in the coronary arteries of KD patients in GSE64486, with the increased abundances of B cells and CD8+ T cells being particularly significant. Combined with the significant decrease in adaptive immune cells seen in the peripheral blood of acute KD indicates that adaptive immune cells, especially B cells and CD8+ T cells, are selectively recruited into the coronary arteries in acute KD. This result also provides a theoretical basis for anti-T cell therapies, such as tacrolimus and the calcineurin inhibitor cyclosporine [73,74], which can quickly control inflammation and are expected to become an effective choice for refractory KD.

We also explored the clinical application value of immune cells in KD by looking for peripheral blood immune cells that can be used to diagnose KD or predict the response to IVIG. We found that the changes in acute KD peripheral blood immune cells were similar to those seen in febrile controls but more dramatic. Among the changes, the increase in neutrophils was specific for KD compared with other febrile illnesses, which indicates that neutrophils may be used as a biomarker to assist the diagnosis of KD. Clinically, IVIG reduces the prevalence of coronary artery abnormalities by reducing tissue inflammation and immune activation [40,75]. Patients with IVIG resistance are at higher risk of developing CAA, and other treatments are needed to control inflammation [76]. Therefore, finding biomarkers that can predict IVIG resistance is an important area of current KD research. Neutrophils [14,77-81], B cells [23], CD8+ T cells [82], and Th17 cells [32,33] in KD peripheral blood have all been reported to predict IVIG resistance, but the types of immune cells supported by different studies are not consistent. Our research did not identify immune cells that can accurately predict IVIG response in multiple datasets. Therefore, the use of immune cell biomarkers to predict IVIG response still needs further exploration and more data verification.

In short, we used public databases to build an atlas that can comprehensively and accurately describe the dynamic changes in the proportions and abundances of KD peripheral blood immune cells. This research is undoubtedly an important basis for studying the immune mechanism of KD. In addition, the bioinformatics research template we use in this study is highly reproducible and can be widely used in immune research on other diseases (such as cancers) using peripheral blood or tissues.

However, this study has some limitations. First, this study is a descriptive study and did not involve the study of the specific biological functions and internal mechanisms of KD peripheral blood immune cells. Second, the coronary artery samples were all from autopsy samples, and the sample size was small, which may affect the accuracy of the results. For example, our study did not show that monocytes and macrophages infiltrate the KD coronary arteries, but they have been reported to do so in the literature, and the abundance of monocytes in this study was even significantly decreased. Fortunately, with the increasing amount of public data and relevant clinical information, the pathological changes and clinical value of immune cells in KD will be more deeply understood. In recent years, single-cell sequencing has developed rapidly, and major breakthroughs have been made in the study of the mechanism of other vascular diseases [83,84]. Such advances are expected to become an important piece in the field of KD immune research.

Acknowledgements

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Tables

Table 1. Basic information of the KD datasets

Datasets	Type and number	Sample source	Year
GSE9863	Acute KD (n=20) and convalescent KD (n=20)	Whole blood	2007
GSE9864	Acute KD (n=53)	Whole blood	2007
GSE18606	Normal (n=9), acute KD (n=20), and convalescent KD (n=19)	Whole blood	2009
GSE63881	Acute KD (n=171) and convalescent KD (n=170)	Whole blood	2014
GSE68004	Normal (n=37), cKD (n=76), inKD (n=13), HAdV (n=19), and GAS (n=14)	Whole blood	2016
GSE73461	Normal (n=55), KD (n=77), DB (n=52), DV (n=94), and ID (n=84)	Whole blood	2018
GSE73463	Acute KD (n=146) and convalescent KD (n=87)	Whole blood	2018
GSE64486	Normal (n=7) and KD (n=8)	Coronary artery	2015

Normal controls in GSE64486 had normal coronary artery histology. Abbreviations: KD, Kawasaki disease; cKD, complete KD; inKD, incomplete KD; HadV, adenovirus; GAS, group A streptococcal disease; DB, definite bacterial infection; DV, definite viral infection; ID, inflammatory diseases.

Table 2. Comparison of peripheral blood immune cell abundances between normal controls, acute KD samples and febrile controls (ImmuCellAI)

Immune Cells	GSE68004	GSE68004	GSE68004	GSE68004	GSE68004	GSE68004	GSE73461	GSE73461	GSE73461	GSE73461	G
	Normal (n=37)	cKD (n=76)	inKD (n=13)	HadV (n=19)	GAS (n=14)	*p* value	Normal (n=55)	KD (n=77)	DB (n=52)	DV (n=94)	I (
B cell	0.185	0.151	0.139	0.147	0.159	0.035	0.226	0.170	0.179	0.204	0
CD4 T	0.160	0.113	0.108	0.142	0.162	<0.001	0.211	0.166	0.170	0.190	0
CD8 T	0.336	0.186	0.198	0.300	0.238	<0.001	0.384	0.274	0.268	0.331	0
CD4 naive	0.041	0.007	0.011	0.026	0.013	<0.001	0.109	0.074	0.094	0.095	0
iTreg	0.097	0.099	0.079	0.073	0.073	0.389	0.174	0.134	0.138	0.168	0
nTreg	0.084	0.071	0.061	0.076	0.069	0.287	0.300	0.299	0.284	0.286	0
Tr1	0.210	0.180	0.153	0.239	0.203	0.022	0.082	0.083	0.063	0.075	0
Th1	0.220	0.140	0.139	0.183	0.169	<0.001	0.191	0.147	0.178	0.191	0
Th2	0.178	0.193	0.128	0.230	0.223	0.060	0.140	0.173	0.189	0.174	0
Th17	0.169	0.351	0.395	0.196	0.237	<0.001	0.417	0.394	0.417	0.432	0
Tfh	0.078	0.040	0.064	0.065	0.070	<0.001	0.056	0.043	0.034	0.044	0
Tcm	0.226	0.124	0.120	0.215	0.167	<0.001	0.182	0.150	0.141	0.186	0
Tem	0.018	0.010	0.005	0.012	0.014	0.037	0.101	0.108	0.107	0.107	0
CD8 naive	0.205	0.142	0.097	0.190	0.153	<0.001	0.164	0.114	0.122	0.123	0
Cytoxic T	0.020	0.023	0.037	0.039	0.033	0.032	0.162	0.193	0.196	0.193	0
Exhausted T	0.028	0.034	0.052	0.029	0.037	0.723	0.003	0.004	0.013	0.004	0

Immune Cells	GSE68004	GSE68004	GSE68004	GSE68004	GSE68004	GSE68004	GSE73461	GSE73461	GSE73461	GSE73461	GSE73461
MAIT	0.157	0.129	0.121	0.144	0.138	0.236	0.227	0.212	0.201	0.222	
Gamma delta T	0.152	0.072	0.083	0.144	0.134	<0.001	0.284	0.235	0.212	0.258	
NKT	0.083	0.170	0.157	0.142	0.146	<0.001	0.155	0.204	0.169	0.165	
NK	0.292	0.228	0.200	0.262	0.239	<0.001	0.287	0.239	0.248	0.250	
Dendritic cell	0.498	0.556	0.507	0.499	0.515	0.308	0.318	0.359	0.326	0.356	
Monocyte	0.154	0.238	0.261	0.229	0.209	<0.001	0.259	0.370	0.365	0.338	
Macrophage	0.115	0.148	0.166	0.122	0.136	0.008	0.208	0.264	0.253	0.198	
Neutrophil	0.118	0.251	0.281	0.150	0.195	<0.001	0.245	0.385	0.360	0.298	
Infiltration Score	0.744	0.748	0.745	0.741	0.742	0.941	0.748	0.779	0.759	0.757	

The above immune cell abundances are average values. Abbreviations: KD, Kawasaki disease; cKD, complete KD; inKD, incomplete KD; HadV, adenovirus; GAS, group A streptococcal disease; DB, definite bacterial infection; DV, definite viral infection; ID, inflammatory diseases.

Table 3. Changes in peripheral blood immune cells in KD

Immune cells	Reported data	References	References
B cells	* The number and relative percentage of B lymphocytes in the peripheral blood of acute KD patients is increased. * After IVIG, the percentage of B cells is decreased. * In patients with acute KD, circulating IgA B lymphocytes and plasma cells are reduced. * There are decreased CD5+ B cells in acute KD. * In acute KD samples, the absolute count of CD23+ B lymphocytes and the ratio of CD23+ B lymphocytes to B lymphocytes are high compared to those in convalescent KD samples and control subjects.	[12,16,17,20-23,46-53] [17,20,22,24] [25,26,54] [22,55] [56]	[12,16,17,20-23,46-53] [17,20,22,24] [25,26,54] [22,55] [56]

Immune cells	Reported data	References	References
T cells	* There are decreased total T cells during the acute phase of KD. * The absolute number of circulating T lymphocytes (CD3+ lymphocytes) in acute KD is significantly decreased and increases after IVIG treatment. * In acute KD, the absolute value of CD4+ T cells is elevated. * The frequencies of CD4+ T cells in the peripheral blood of patients with acute KD are significantly lower than those of patients with subacute KD and control children. * The levels of CD4+ T cells in peripheral blood of KD patients are in the normal and low ranges. * There is not a significant increase in the activation of overall peripheral blood CD4+ and CD8+ T cell in KD patients. * After IVIG, the percentages of CD3+ and CD4+ cells do not change. * The absolute CD4+ and CD8+ T cell counts are decreased in acute KD and increased in convalescent KD. * In KD patients, the proportions and markers of Treg are significantly lower than those in healthy donors, while the proportions and gene markers of Tregs are significantly increased after IVIG treatment. * Compared with those in normal controls and febrile controls, the plasma IL-17A (Th17 cells) concentration of KD patients is increased, and IL-17A decreases after IVIG treatment. * Before IVIG, the frequency of cTfh2 cells in KD patients is significantly increased	[57] [25] [22,23] [13,27] [28] [29] [24] [17] [30-33] [30-33,58] [59] [30-33] [24,60] [61]	[57] [25] [22,23] [13,27] [28] [29] [24] [17] [30-33] [30-33,58] [59] [30-33] [24,60] [61]

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Immune cells	Reported data	References	References
NK cells	* The absolute number of NK cells in the acute phase of KD is significantly decreased and increases after IVIG treatment.	[20,23,25,62-64]	[20,23,25,62-64]
DCs	* Myeloid DC (mDC) cells are increased in the peripheral blood of patients with acute KD and decline in the subacute phase.	[58]	[58]
Monocytes &Macrophages	* In acute KD, the numbers of peripheral blood CD14+ monocytes/macrophages and activated CD14+CD23+ monocytes/macrophages are increased. * The number of CD14+ CD16+ (FcγRIII) monocytes/macrophages in acute KD is increased and decreases with IVIG treatment. * Immunocytochemical and immunoelectron microscopic studies have shown that monocytes partly differentiate into macrophages in acute KD peripheral circulation.	[16,65] [17,66] [67,68]	[16,65] [17,66] [67,68]

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Immune cells	Reported data	References	References
Neutrophils	* Neutrophils are significantly increased in acute KD and decreased in convalescent KD. * Compared with that in febrile controls, the number of neutrophils in patients with acute KD is increased, and these neutrophils are also activated, as evidenced by their expression of various late-stage granulocyte-specific genes. * Compared with patients with acute adenovirus infection or systemic adverse reactions, KD patients have higher expression levels of neutrophil activation-related genes, but the levels are not higher than those of scarlet fever patients.	[12-15] [69,70] [71]	

Figure Legends

Figure 1. The inflammatory response is the most significant hallmark of Kawasaki disease (KD). (A) Verified differentially expressed genes (VDEGs) in the KD datasets GSE18606, GSE68004, and GSE73461. (B) Top 20 Metascape enrichment analysis results of VDEGs. (C) Metascape enrichment network showing the similarity of enrichment terms within and between the top 20 clusters. (D) Gene set enrichment analysis (GSEA) of the top 10 significant hallmarks in KD samples.

Figure 2. Atlas of circulating immune cells in acute and convalescent KD. Proportions of 22 types of immune cells in (A) normal controls, (B) acute KD samples, and (C) convalescent KD samples based on CIBERSORT. (D) Changes in the proportions of innate immune cells and adaptive immune cells during the progression and regression of KD. (E) Map showing the differences in KD immune cell proportions (CIBERSORT). The former three columns represent the comparisons of immune cell proportions between acute KD samples and normal controls. The latter three columns represent the comparisons of immune cell proportions between acute KD and convalescent KD samples. (F) Map showing the differences in KD immune cell abundances (ImmuCellAI). The former three columns represent the comparisons of immune cell abundances between the acute KD group and the normal group. The latter 3 columns represent the comparisons of immune cell abundances between acute KD and convalescent KD samples. The superscripts in (E) and (F) are the names of the datasets, and the color of the square indicates the significance. The deeper red the color is, the higher the abundance or proportion of the immune cell is in acute KD samples compared with normal controls or convalescent KD samples. The blue represents the opposite pattern. NA, not applicable; NS, not significant.

Figure 3. The relationship between the response of KD patients to intravenous immunoglobulin (IVIG) and the levels of peripheral blood immune cells. (A) Sankey diagram of the relationship between IVIG

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response and aneurysm formation in GSE63881. (B) The former two columns represent the comparisons of immune cell proportions (CIBERSORT) between IVIG responders and IVIG nonresponders in acute KD. The latter two columns represent the comparisons of immune cell proportions between IVIG responders and IVIG nonresponders in convalescent KD. (C) The former two columns represent the comparisons of immune cell abundances (ImmuCellAI) between IVIG responders and IVIG nonresponders in acute KD. The latter two columns represent the comparisons of immune cell abundances between IVIG responders and IVIG nonresponders in convalescent KD. (D) The former three columns represent the comparisons of immune cell abundances (immune cell markers) between IVIG responders and IVIG nonresponders in acute KD. The latter two columns represent the comparisons of immune cell abundances between IVIG responders and IVIG nonresponders in convalescent KD. The superscripts are the names of the datasets, and the color of the square indicates the significance. The deeper red the color is, the higher the abundance or proportion of the immune cell is in IVIG responders compared with IVIG nonresponders. The blue represents the opposite pattern. NA, not applicable; NS, not significant.

Figure 4. Comparison of immune cell abundances in coronary arteries between normal controls and KD samples (ImmuCellAI).

Figure 5. Schematic diagram of KD pathogenesis. The high-incidence period of KD is between 6 months and 5 years of age, which coincides with the immune trough period when maternal immunity declines and the child's own immune system is immature. Nonspecific pathogens infect genetically susceptible children through the respiratory tract, and the most harmful effect of the systemic immune response induced by these pathogens is coronary vasculitis. Necrotizing arteritis occurs in the first two weeks of KD and is manifested by neutrophils infiltrating the coronary arteries. This effect is followed by inflammatory infiltration of monocytes, macrophages, B cells, CD4+ T cells, and CD8+ T cells during the period of subacute chronic arteritis. However, in acute KD, adaptive immune cells such as B cells, CD4+ T cells and CD8+ T cells in the peripheral blood are significantly decreased, which suggests that adaptive immune cells are selectively recruited from the peripheral circulation to KD target tissues.

Supplementary Information

Table S1. Comparison of peripheral blood immune cell abundances between normal controls and acute KD samples (ImmuCellAI).

Table S2. Comparison of peripheral blood immune cell abundances between acute KD and convalescent KD samples (ImmuCellAI).

Figure S1. Summary of the enrichment analysis in DisGeNET.

Figure S2. Comparison of immune cell proportions (CIBERSORT) between acute KD samples and normal controls in (A) GSE18606, (B) GSE68004, and (C) GSE73461.

Figure S3. Comparison of immune cell proportions (CIBERSORT) between acute KD and convalescent KD samples in (A) GSE18606, (B) GSE63881, and (C) GSE73463.

Figure S4. Comparison of immune cell abundances (ImmuCellAI) between acute KD samples and normal controls in (A) GSE18606, (B) GSE68004, and (C) GSE73461.

Figure S5. Comparison of immune cell abundances (ImmuCellAI) between acute KD and convalescent KD samples in (A) GSE9863, (B) GSE18606, (C) GSE63881, and (D) GSE73463.

Figure S6. Map of the differences in KD immune cell abundances (as determined based on immune cell markers). The former three columns represent the comparisons of immune cell abundances between acute KD samples and normal controls. The latter 4 columns represent the comparisons of immune cell abundances between acute KD and convalescent KD samples.

Figure S7. Comparison of immune cell abundances (determined based on immune cell markers) between acute KD samples and normal controls in (A) GSE18606, (B) GSE68004, and (C) GSE73461.

Figure S8. Comparison of immune cell abundances (determined based on immune cell markers) between acute KD and convalescent KD samples in (A) GSE9863, (B) GSE18606, (C) GSE63881, and (D) GSE73463.

Figure S9. The decrease in CD4+ T cells and CD8+ T cells and the increase in neutrophils in KD peripheral blood are specific. (A) Differences in the proportions of CD8+ T cells and neutrophils between the normal controls, KD samples and febrile controls in GSE68004. (B) Differences in the proportions of CD8+ T cells and neutrophils between the normal controls, KD samples and febrile controls in GSE73461. (C) Differences in the abundances of cytotoxic T cells (quantified with marker CD8A), T helper cells (CD4) and neutrophils (CXCR2) between the normal controls, KD samples and febrile controls in GSE68004. (D) Differences in the abundances of cytotoxic T cells, T helper cells and neutrophils between the normal controls, KD samples and febrile controls in GSE73461. Abbreviations: cKD, complete KD; InKD: incomplete KD; AdV: adenovirus; GAS: group A Streptococcus. *p<0.05, ** p<0.01, *** p<0.001, ns: not significant.

Figure S10. The abundance of neutrophils in peripheral blood can be used as a biomarker for KD diagnosis. (A) The ROC curve of differential diagnosis between KD samples and normal controls, HAdV samples, and GAS samples in GSE68004. (B) The ROC curve of differential diagnosis between KD samples and normal controls, DB samples, DV samples and ID samples in GSE73461. Abbreviations: KD, Kawasaki disease; cKD, complete KD; inKD, incomplete KD; HadV, adenovirus; GAS, group A Streptococcus; DB, definite bacterial infection; DV, definite viral infection; ID, inflammatory disease; ROC, receiver operating characteristic; AUC, area under the ROC curve.

Figure S11. Comparison of immune cell proportions (CIBERSORT) between IVIG responders and IVIG nonresponders in (A) GSE18606 acute KD, (B) GSE63881 acute KD, (C) GSE18606 convalescent KD, and (D) GSE63881 convalescent KD samples.

Figure S12. Comparison of immune cell abundances (ImmuCellAI) between IVIG responders and IVIG nonresponders in (A) GSE18606 acute KD, (B) GSE63881 acute KD, (C) GSE18606 convalescent KD, and (D) GSE63881 convalescent KD samples.

Figure S13. Comparison of immune cell abundances (immune cell markers) between IVIG responders and IVIG nonresponders in (A) GSE9864 acute KD, (B) GSE18606 acute KD, (C) GSE63881 acute KD, (D) GSE18606 convalescent KD, and (E) GSE63881 convalescent KD samples.

Figure S14. The relationship between aneurysms and the level of peripheral blood immune cells in KD. (A) Comparison of immune cell proportions (determined based on CIBERSORT) between KD patients with coronary artery aneurysm, dilated coronary artery and normal coronary artery. (B) Comparison of immune cell abundances (determined based on ImmuCellAI) between KD patients with coronary artery aneurysm, dilated coronary artery and normal coronary artery. (C) Comparison of immune cell abundances (determined based on immune cell markers) between KD patients with coronary artery aneurysm, dilated coronary artery and normal coronary artery.

Figure S15. Comparison of the immune cell abundances in coronary arteries between normal controls and KD samples (determined based on immune cell markers). *p<0.05, ** p<0.01, *** p<0.001, ns: not significant

A

GSE18606 up | GSE68004 up
GSE73461 up

4053 | 633 | 1039
214 | 1546 | 1203
448

GSE18606 down | GSE68004 down
GSE73461 down

3121 | 800 | 676
440 | 1453 | 963
922

B

GO:0002274: myeloid leukocyte activation
GO:0002521: leukocyte differentiation
R-HSA-2262752: Cellular responses to stress
GO:0002253: activation of immune response
R-HSA-1280218: Adaptive Immune System
GO:0010942: positive regulation of cell death
R-HSA-449147: Signaling by Interleukins
GO:0030099: myeloid cell differentiation
GO:0031349: positive regulation of defense response
GO:0022613: ribonucleoprotein complex biogenesis
GO:0001819: positive regulation of cytokine production
GO:0002683: negative regulation of immune system process
GO:0097190: apoptotic signaling pathway
R-HSA-109582: Hemostasis
GO:0019058: viral life cycle
GO:0050900: leukocyte migration
GO:0043043: peptide biosynthetic process
GO:0006979: response to oxidative stress
GO:0006914: autophagy
GO:0046631: alpha-beta T cell activation

-log10(P)

C

myeloid leukocyte activation
leukocyte differentiation
Cellular responses to stress
activation of immune response
Adaptive Immune System
positive regulation of cell death
Signaling by Interleukins
myeloid cell differentiation
positive regulation of defense response
ribonucleoprotein complex biogenesis
positive regulation of cytokine production
negative regulation of immune system process
apoptotic signaling pathway
Hemostasis
viral life cycle
leukocyte migration
peptide biosynthetic process
response to oxidative stress
autophagy
alpha-beta T cell activation

D

Enrichment Score

Hallmarks	NES	FDR
Inflammatory response	2.36	<0.001
Epithelial mesenchymal transition	2.34	<0.001
TNFA signaling via NFKB	2.31	<0.001
IL6-JAK-STAT3 signaling	2.29	<0.001
Coagulation	2.27	<0.001
Hypoxia	2.26	<0.001
Reactive oxygen species pathway	2.18	<0.001
KRAS signaling up	2.16	<0.001
Xenobiotic metabolism	2.15	<0.001
Complement	2.10	<0.001

KD<----------->Normal

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