Expression of Eosinophilic Subtype Markers in Patients with Kawasaki Disease

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Abstract: Purpose: Eosinophils may rise to a higher level in the acute phase of Kawasaki disease (KD) both before and after intravenous immunoglobulin (IVIG) therapy. A substantial body of research was carried out on the association between KD and allergic diseases. Eosinophils play an important role in type 2 inflammation. Recent studies have shown that there are two distinct subtypes of eosinophils. In addition to their role in inflammation, lung-resident eosinophils (rEOS) also regulate homeostasis. Inflammatory eosinophils (iEOS) reflect type 2 inflammation in tissues. IEOS were considered the primary eosinophils in non-severe allergic asthma, while rEOS were thought to be the primary eosinophils in severe non-allergic eosinophilic asthma. This case–control study aimed to investigate the marker expression of eosinophilic subtypes in KD patients. Materials and Methods: The marker expressions of eosinophilic subtypes in the leukocytes of patients with KD were evaluated by the recently established KDmarkers online tool, a web server including gene expression data. Results: In our screening tools, we found that the gene IL10RA was significantly higher after half a year compared to febrile controls. Conclusions: To our knowledge, this is the first study to demonstrate that KD patients have increased SELL than febrile controls after 6 months of treatment. We present evidence here that dynamically different eosinophilic involvement exists between KD patients with and without CAL. The role of eosinophilic subtypes in KD patients warrants further investigation.

Keywords: allergy; coronary artery lesions; eosinophils; IVIG; Kawasaki disease

1. Background

Discovered over 60 years ago, Kawasaki disease (KD) has since been one of the major acquired heart diseases with rare life-threatening complications [1]. It is thus an important medical issue to track the long-term health of children with KD, a typically self-limited condition. Studies have found that children with KD are more likely to be hospitalized for infections or allergic diseases [2–4]. In addition to cardiovascular sequelae, KD has been shown to have other long-term effects, leading to common health problems such as allergic diseases and compromised immunity [5,6]. Our latest study of immunoglobulin...
(Ig)M receptors is the first to show that FCMR transcripts decline after six months of KD [6]. The National Health Insurance Research Database shows that KD patients are more likely to develop allergic diseases, such as asthma and rhinitis, after recovery than the control group [7]. Increases in eosinophil cationic protein (ECP), interleukin (IL)-5, eosinophils, and total IgE in children with KD were considered markers of allergy [8,9]. The nasal epithelium is the main entry point into the airways. At mucosal sites, plasma cells produce IgA dimers that neutralize pathogens and particles. A recent study has shown that IgA levels have been related to coronary artery lesions (CAL) in KD [10]. Eosinophils are required to generate and maintain mucosal IgA plasma cells [11]. Eosinophils play important roles in type 2 inflammation, host immune defense against infection, and various biological functions including beneficial effects, immune regulation, and homeostasis [12–14]. Early studies showed that eosinophil levels were highly elevated in the acute phase of KD before and after intravenous immunoglobulin (IVIG) therapy [9]. Tsai et al. demonstrated that eosinophils > 1.5% may be helpful in diagnosing KD in other febrile patients [15]. Liu’s findings suggest that eosinophilia may be an important motivating factor in the diagnosis of KD [16]. The highest percentage of eosinophils in the convalescent phase after IVIG has long been recognized [17]. Eosinophilia (≥4%) after IVIG treatment was detected as a predictor of IVIG responsiveness and the Z-score of eosinophils was detected as a predictor of CAL [9,18]. Eosinophils were found to accumulate in epicardial macroangioses in the autopsy of four KD patients [19].

Recent findings highlighted that multiple functions of eosinophils may be attributed to the subpopulation [20,21]. Marked phenotypic heterogeneity included surface markers, anatomical location, response to IL-5 and nuclear morphology. Transcriptional profiling of mouse eosinophils identified distinct gene signatures. However, subpopulations of eosinophils were largely unknown in human diseases. Lung-resident eosinophils (rEOS) in the steady-state normal lung also regulate homeostasis, termed homeostatic eosinophils, Siglec-F^{int}CD62L^{+}CD101^{low} cells with a ring-shaped nucleus [20,22,23]. The predominant eosinophil in non-severe allergic asthma is inflammatory (iEOS), whereas the predominant eosinophil in severe non-allergic eosinophilic asthma is the rEOS [24]. iEOS recruited after being challenged with house dust mites in mice are IL-5-dependent, defined as SiglecF^{high}CD62L^{−}CD101^{high} cells with segmented nuclei [23]. Transcriptomic profiles showed the alpha subunit of the IL10RA-encoding IL-10 receptor as a significantly and differentially expressed gene between iEOS and rEOS [20]. Compared with sibling controls, children with KD and without CAL developed allergic rhinitis and any other exacerbated forms of allergy. This finding is consistent with the previously observed lower T helper (Th)2 cytokines in KD patients with CAL [8]. Whether pro-inflammatory receptor P2 and anti-inflammatory receptor P1 behave differently has not been resolved [13]. Matucci et al. found a high proportion of inflammatory eosinophils in patients with severe asthma, especially in nasal polyps [25].

The final product of rEOS is L-selectin encoded by SELL, also known as CD62L in which the recovery phase of KD tends to be higher than the acute or subacute phase [26]. Plasma L-selectin levels tend to be lower in patients with CAL than in patients without CAL. This finding is consistent with decreased eosinophils in CAL patients. Eosinophilic chronic sinusitis (ECRS) is a type of rhinitis characterized by prominent eosinophilic infiltration. L-selectin plays a role in eosinophil recruitment in ECRS human nasal mucosa [27]. However, there is little ongoing research or available information on the eosinophilic subtypes of KD. This case–control study was designed to examine whether the expression of different markers of eosinophils is altered in children with KD.

2. Materials and Methods

mRNA expression profiles were obtained from leukocyte samples collected during a previously published experiment and uploaded to the NCBI GEO database (GSE109351 series); all methods and materials used to obtain data are provided in the cited publications from a total of 50 KD patients before IVIG and 18 KD patients three weeks after IVIG,
while 18 non-febrile and 18 febrile controls were included in Human Transcriptome Array analysis (Table 1) [6,28,29]. Each sample was pooled from six patients (Figure 1). Marker expression of eosinophilic subtypes in leukocytes of KD patients was assessed using the recently established KDmarkers online tool (https://cosbi.ee.ncku.edu.tw/KDmarkers/, accessed on 13 January 2022), a web server that contains gene expression data of the GeneChip Human Transcriptome Array 2.0 (HTA 2.0, Affymetrix, Santa Clara, CA, USA) covering more than 285,000 full-length transcripts [29]. In order to identify the significant ratio of transcriptome level and 1/ratio (fold changes), one-way analysis of variance was conducted by the default setting of the commercial microarray tool Partek. Finally, the results were checked and proven by quantitative reverse transcriptase polymerase chain reaction (RT-PCR).

Table 1. Demographic characteristics of the subjects for establishing KDmarkers online tool.

|                      | Non-Febrile Controls (n = 18) | Febrile Controls (n = 18) | KD before IVIG (n = 50) | KD after IVIG (n = 18) |
|----------------------|-------------------------------|---------------------------|--------------------------|------------------------|
| Age (years)          | 2.46 ± 0.35                  | 1.97 ± 0.26               | 1.36 ± 0.14              | 1.90 ± 0.32            |
| Male/female ratio    | 1                             | 0.8                       | 1.17                     | 1                      |

IVIG, intravenous immunoglobulin; KD, Kawasaki disease.

Figure 1. Cont.
The first venous blood sample was collected from each patient on the first day of diagnosis or before IVIG therapy (KD1). Samples were obtained 3 to 7 days and 3 weeks post-treatment (KD2/KD3) following acute and subacute IVIG treatment. KD4 was defined as six months after IVIG. We measured the lumen diameter of the coronary arteries using two-dimensional echocardiography. CAL was defined as coronary findings in KD patients and controls. All KD patients were admitted to the hospital with acute infection.

Demographic characteristics of the subjects for establishing KDmarkers online tool.

Table 1. mRNA Detected by the Quantitative Reverse Transcriptase-Polymerase Chain Reaction

The study employed a retrospective case–control study in a single center. We used RT-PCR to validate the mRNA levels of SELL and IL10RA in KD patients and controls. All patients with fever plus ≥ 4 of 5 clinical criteria (extremity changes, rash, conjunctivitis, oral changes, and cervical lymphadenopathy) or fever plus fewer principal criteria and coronary artery abnormalities identified by echocardiography met the diagnostic criteria for KD specified by the American Heart Association after excluding other febrile illnesses [30]. This study was approved by the Institutional Review Board (IRB) of Kaohsiung Chang Gung Memorial Hospital (202002142A3) and carried out according to the Declaration of Helsinki. After a full description of the study, all parents or guardians signed informed consent. All KD patients were admitted to the pediatric ward of Chang Gung Memorial Hospital in Kaohsiung, Taiwan and received a single high-dose IVIG of 2 g/kg each time. The first venous blood sample was collected from each patient on the first day of diagnosis or before IVIG therapy (KD1). Samples were obtained 3 to 7 days and 3 weeks post-treatment (KD2/KD3) following acute and subacute IVIG treatment. KD4 was defined as six months after IVIG. We measured the lumen diameter of the coronary arteries using two-dimensional echocardiography. CAL was defined as coronary findings in KD patients meeting the following criteria: ≥3.0 mm in inner diameter in children under 5 years old, ≥4.0 mm in inner diameter in children over 5 years old, a diameter ≥1.5 times greater than that of an adjacent segment, and irregular luminal contour according to the guidelines of the Japanese Ministry of Health or a z-score of ≥2.5 [31,32]. Patients whose symptoms did not meet the KD criteria were excluded. Age- and sex-matched febrile patients with no history of KD were studied as controls for comparison. The fever-controlled patients were admitted to the hospital with acute infection.

Genes validated in leukocytes were selected to be associated with rEOS (SELL) and iEOS (IL10RA). Sequence information for all primers used were: SELL, FP—CAGTCTACCTGCAGCACAGC; RP—TGGGTTGCTCTGACATTTCC; IL10RA, FP—CCCTGTCTATGACCT
TACCG; RP—CACACTGCAAATGTACAGGT; 18s, FP—GTAACCCGTTGAAACCCAT; RP—CCATCCAATCGGTAGTAGCG. Final data were normalized using the geometric mean of housekeeping genes, 18s. Total RNA from peripheral leukocytes was extracted from cell lysates using a miRNA isolation kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. An equal amount of 1 µg of RNA was then reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo, Waltham, MA, USA). RT-PCR was performed in triplicate on a 7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) using Fast SYBR Green PCR Master Mix (Applied Biosystems, Thermo, Waltham, MA, USA). Relative amounts of all mRNAs were normalized to 18s mRNA expression levels using the $2^{-\Delta\Delta CT}$ method; results are shown as fold changes relative to controls.

Continuous data in results, tables, and figures are presented as mean and standard error. We evaluated quantitative data between controls and KD or between KD patients with CAL and without CAL with the Mann–Whitney U test. We performed the necessary chi-square or Fisher’s exact test on categorical data. We applied all statistical analyses using SPSS version 12.0 for Windows (IBM, New York, USA), and we considered two-sided tests with a $p$-value of $< 0.05$ to be of statistical significance.

3. Results

Representative datasets that have been detailed with fold changes and $p$-values are summarized in Table 2. We found that the representative gene SELL for rEOS was significantly higher in KD (Figure 1) [20]. In the different mRNA expressions of rEOS and iEOS in the transcriptome arrays below, we set the cutoff criteria to be all $p$-values $< 0.01$ in KD before IVIG compared to non-febrile controls, febrile controls and three weeks after IVIG [20].

rEOS, PDE2A, LAIR1, SELL, PRG3, RUNX3, P2RX4, IL12RB2, METRN1, PON2, NEDD4, ANXA1, LDLR, MYC, S1PR2, SERPINB1, Rnase1, Rnase3, Rnase12 (Table 2A).

iEOS, CD34, CD101, RIPK2, HCAR2, ITGA1, ITGAX, TLR4, BCL2A1, IL13RA1, IL1RN, SLC3A2, TREM1, C3AR1, HIF0, CD300C (AF251705), MIF, OLFM4, GRN, LPIN1, CD33, IL10RA, RETN, ACP5, CXCR2, IL6, ADORA2A, BCL6, PGF (Table 2B).

Figure 1A–E show that the KDmarkers online tool (https://cosbi.ee.ncku.edu.tw/KDmarkers/, accessed on 13 January 2022) identified three genes (lower IL10RA, higher SELL, and SERPINB1) and indicated the role of rEOS after filtering by the cut-off criteria we set. In contrast, the iEOS representative gene CD101 was not elevated in KD (Table 2B).

To sum up the salient features of the analysis, several findings are worth noting. In this study, we examined the mRNA expression of the above 28 iEOS markers in healthy controls (HC), febrile controls (FC), acute KD (KD1), and KD patients (KD3) 21 days after IVIG treatment (Figure 1). It was found that, among the 28 iEOS markers examined, 9 markers (namely HCAR2, ITGAX, TLR4, C3AR1, OLFM4, GRN, CD33, RETN, and BCL6) had significantly higher mRNA expression in the acute phase of KD compared with HC (Table 2). IVIG treatment resulted in a significant reduction in the facet of 12 genes, including HCAR2, ITGAX, TLR4, IL1RN, C3AR1, OLFM4, GRN, CD33, RETN, CXCR2, and BCL6. The expressions of LPIN1 and IL10RA were lower in the acute phase of KD and were significantly increased after IVIG treatment.

We also examined the mRNA expression of 18 rEOS markers in KD patients and controls (Table 1). Of the 18 rEOS markers observed, 6 markers (LAIR1, SELL, NEDD4, LDLR, SERPINB1, and Rnase3) significantly increased mRNA expression in acute phase KD and then significantly decreased after IVIG treatment. One marker, RUNX3, decreased mRNA expression in the acute phase of KD and then increased after IVIG treatment. SELL between KD and controls was significantly different in the transcriptome array (Figure 1). The results after IVIG showed that SELL was significantly lower than that of KD patients in the pre-IVIG group.
Table 2. (A) Molecular biomarker of lung-resident (Table 2A) and inflammatory (Table 2B) eosinophils for Kawasaki disease by transcripts expressions; (B) Transcript expressions of inflammatory eosinophils between Kawasaki disease patients and control subjects.

| Gene Symbol | RefSeq  | Column ID       | Fold-Change (KD1 vs. HC) | p-Value (KD1 vs. HC) | Fold-Change (KD1 vs. FC) | p-Value (KD1 vs. FC) | Fold-Change (KD3 vs. KD1) | p-Value (KD3 vs. KD1) |
|-------------|---------|-----------------|--------------------------|----------------------|--------------------------|----------------------|--------------------------|------------------------|
| PDE2A       | NM_001143839 | TC11002059.hg.1 | 1.133                    | 0.223                | 1.074                    | 0.474                | 1.040                    | 0.743                  |
| LAIR1       | NM_002287 | TC19001844.hg.1 | 1.386                    | 0.001 **             | 1.130                    | 0.108                | −1.421                   | <0.001 **              |
| SELL        | NM_000655 | TC10003500.hg.1 | 1.355                    | 0.005 **             | 1.312                    | 0.009 **             | −1.484                   | 0.001 **               |
| PRG3        | NM_006093 | TC11001790.hg.1 | 1.035                    | 0.718                | −1.046                   | 0.634                | 1.032                    | 0.739                  |
| RUNX3       | NM_001031680 | TC10002570.hg.1 | −1.274                   | 0.004 **             | −1.224                   | 0.011 *              | 1.245                    | 0.007 **               |
| P2RX4       | NM_002560 | TC12000856.hg.1 | 1.010                    | 0.864                | −1.148                   | 0.033 *              | 1.025                    | 0.686                  |
| IL12RB2     | NM_001559 | TC10000741.hg.1 | −1.046                   | 0.537                | −1.247                   | 0.013 *              | −1.116                   | 0.154                  |
| MATERNAL    | NM_001004431 | TC17000959.hg.1 | −1.026                   | 0.715                | 1.024                    | 0.739                | 1.019                    | 0.788                  |
| PON2        | NM_000305 | TC07001612.hg.1 | −1.076                   | 0.395                | −1.081                   | 0.369                | 1.057                    | 0.519                  |
| NEDD4       | NM_006154 | TC15001471.hg.1 | 1.452                    | 0.031 *              | 1.424                    | 0.038 *              | −1.441                   | 0.033 *                |
| ANXA1       | NM_000700 | TC09000339.hg.1 | 1.415                    | 0.092                | 1.709                    | 0.018 *              | −1.525                   | 0.049 *                |
| LDLR        | NM_000527 | TC19000191.hg.1 | 1.322                    | 0.017 *              | 1.016                    | 0.870                | −1.418                   | 0.005 *                |
| MYC         | NM_002467 | TC08000749.hg.1 | −1.061                   | 0.334                | −1.191                   | 0.016 *              | 1.116                    | 0.093                  |
| S1PR2       | NM_004230 | TC19001160.hg.1 | −1.099                   | 0.489                | −1.218                   | 0.168                | 1.117                    | 0.417                  |
| SERPINB1    | NM_030666 | TC06001217.hg.1 | 1.955                    | 0.001 **             | 1.736                    | 0.004 **             | −2.036                   | <0.001 **              |
| Rnase1      | NM_002933 | TC14000902.hg.1 | 1.088                    | 0.367                | −1.099                   | 0.316                | −1.046                   | 0.624                  |
| Rnase3      | NM_002935 | TC14000027.hg.1 | 2.016                    | 0.002 *              | 1.475                    | 0.039 *              | −1.836                   | 0.005                  |
| Rnase12     | NM_001024822 | TC14000890.hg.1 | −1.008                   | 0.854                | −1.045                   | 0.310                | 1.014                    | 0.745                  |
Table 2. Cont.

| Gene Symbol | RefSeq       | Column ID                                    | (B) Fold-Change (KD1 vs. HC) | p-Value (KD1 vs. HC) | Fold-Change (KD1 vs. FC) | p-Value (KD1 vs. FC) | Fold-Change (KD3 vs. KD1) | p-Value (KD3 vs. KD1) |
|-------------|--------------|----------------------------------------------|------------------------------|----------------------|--------------------------|----------------------|---------------------------|----------------------------|
| CD34        | NM_001025109 | TC01003777.hg.1                             | 1.005                        | 0.949                | -1.010                   | 0.905                | 1.052                     | 0.537                      |
| CD101       | NM_001256106 | TC01001025.hg.1                             | 1.183                        | 0.097                | 1.006                    | 0.946                | -1.202                    | 0.074                      |
| RIPK2       | NM_003821    | TC08000545.hg.1                             | -1.010                       | 0.961                | 1.523                    | 0.071                | -1.056                    | 0.793                      |
| HCAR2       | NM_177551    | TC12002078.hg.1                             | 2.185                        | 0.025 *              | 1.328                    | 0.345                | -2.924                    | 0.005 **                   |
| ITGA1       | NM_181501    | TC05003439.hg.1                             | 1.100                        | 0.334                | 1.083                    | 0.413                | -1.283                    | 0.027 *                    |
| ITGAX       | NM_000887    | TC16000375.hg.1                             | 1.774                        | 0.009 **             | 1.578                    | 0.026 *              | -1.712                    | 0.012 *                    |
| TLR4        | NM_003266    | TC09000601.hg.1                             | 1.858                        | 0.019 *              | 1.658                    | 0.044 *              | -2.145                    | 0.007 **                   |
| BCL2A1      | NM_00114735  | TC15001719.hg.1                             | 1.242                        | 0.144                | 1.615                    | 0.007 **             | -1.348                    | 0.057                      |
| IL13RA1     | NM_001560    | TC0X000575.hg.1                             | 1.294                        | 0.185                | 1.380                    | 0.108                | -1.426                    | 0.081                      |
| IL1RN       | NM_173843    | TC02000720.hg.1                             | 1.465                        | 0.054                | 1.222                    | 0.271                | -1.657                    | 0.018 *                    |
| SLCSA2      | NM_001012662 | TC11000567.hg.1                             | -1.160                       | 0.057                | -1.281                   | 0.006 **             | 1.182                     | 0.036 *                    |
| TREM1       | NM_001242589 | TC06001717.hg.1                             | -1.027                       | 0.888                | 1.250                    | 0.252                | -1.047                    | 0.807                      |
| C3AR1       | NM_004054    | TC12001171.hg.1                             | 3.061                        | 0.003 **             | 1.172                    | 0.577                | -2.732                    | 0.006 **                   |
| HIF0        | NM_005318    | TC22000288.hg.1                             | 1.103                        | 0.293                | -1.055                   | 0.555                | -1.089                    | 0.354                      |
| CD300C      | (AF251705)   | TC09000601.hg.1                             | 1.205                        | 0.176                | -1.079                   | 0.562                | -1.195                    | 0.193                      |
| MIF         | NM_002415    | TC22001456.hg.1                             | -1.119                       | 0.402                | -1.182                   | 0.225                | 1.018                     | 0.892                      |
| OLFM4       | NM_006418    | TC13000228.hg.1                             | 2.604                        | 0.023 *              | 1.253                    | 0.527                | -2.573                    | 0.024 *                    |
| GRN         | NM_002057    | TC17000570.hg.1                             | 1.515                        | 0.041 *              | -1.057                   | 0.752                | -1.662                    | 0.018 *                    |
| LP1N1       | NM_145693    | TC02000768.hg.1                             | -1.082                       | 0.037 *              | -1.095                   | 0.021 *              | 1.077                     | 0.046 *                    |
| CD33        | NM_001772    | TC19000775.hg.1                             | 1.197                        | 0.012 *              | 1.042                    | 0.480                | -1.167                    | 0.024 *                    |
| IL10RA      | NM_001558    | TC11001058.hg.1                             | -1.351                       | <0.001 **            | -1.262                   | 0.002 **             | 1.300                     | 0.001 **                   |
| RETN        | NM_002041    | TC19000133.hg.1                             | 1.365                        | 0.042 *              | -1.004                   | 0.974                | -1.453                    | 0.020 *                    |
| ACP5        | NM_00111034  | TC19001188.hg.1                             | -1.023                       | 0.731                | -1.111                   | 0.133                | 1.007                     | 0.914                      |
| CXCR2       | NM_001168298 | TC02001292.hg.1                             | 1.531                        | 0.066                | 1.306                    | 0.219                | -1.894                    | 0.013 *                    |
| IL6         | NM_000600    | TC07001137.hg.1                             | -1.071                       | 0.337                | 1.077                    | 0.296                | -1.004                    | 0.960                      |
| ADORA2A     | NM_0008675   | TC22014874.hg.1                             | -1.025                       | 0.688                | -1.053                   | 0.375                | 1.121                     | 0.069                      |
| BC6         | NM_001130845 | TC03002969.hg.1                             | 2.047                        | 0.005 **             | 1.675                    | 0.024 *              | -2.072                    | 0.004 **                   |
| PGF         | NM_001207012 | TC14001313.hg.1                             | -1.007                       | 0.951                | -1.018                   | 0.882                | 1.094                     | 0.465                      |

KD1: Kawasaki disease before IVIG treatment; KD3: Kawasaki disease > 3 weeks after IVIG treatment; FC: febrile control; HC: non-febrile control. * indicates p < 0.05 and ** indicates p < 0.01 between the groups.
The results of the array analysis were verified by RT-PCR. SELL mRNA levels were significantly elevated in leukocytes of KD3 ($n = 22$) and KD4 ($n = 20$) patients compared with febrile controls ($n = 24$) (Figure 2 and Table 3). Patients with CAL ($n = 39$) had elevated SELL levels compared with patients without CAL before treatment ($n = 55$) (CAL, $1.33 \pm 0.18$; non-CAL, $0.87 \pm 0.12$; $p = 0.012$). SELL mRNA levels increased significantly in patients without CAL after three weeks and six months compared with controls (see Figure 2). In addition, IL10RA was used for further validation by RT-PCR. However, we found similar IL10RA mRNA levels in the controls and KD patients after 6 months of treatment (see Figure 3 and Table 4). It was observed that levels of IL10RA in leukocytes increased significantly in KD1 ($n = 43$, compared with febrile controls ($n = 32$) (KD1, $2.17 \pm 0.30$; fever control, $1 \pm 0.12$; $p = 0.013$). The IL10RA mRNA expression levels were significantly different between patients with and without CAL before and after IVIG ($p < 0.001$ and $= 0.043$, respectively).

Figure 2. SELL mRNA levels (A) in febrile controls and patients with Kawasaki disease (KD1, before treatment; KD2, 3–7 days after treatment; KD3, three weeks after treatment; KD4, six months after treatment); (B) in KD patients with and without coronary artery lesions (CAL). * denotes significance ($^* p < 0.05$; $^{***} p < 0.001$) between controls and KD patients, and KD patients with and without CAL.
Table 3. (A) Demographic characteristics of the subjects with Kawasaki disease (KD) for SELL mRNA; (B) SELL mRNA levels between patients with and without coronary artery lesions (CAL).

(A)

|                  | Febrile Controls | KD          | p-Value |
|------------------|------------------|-------------|---------|
|                  | (n = 24)         | (n = 94)    |         |
| Age (years)      | 2.14 ± 0.26      | 1.84 ± 0.17 | 0.088   |
| male             | 10               | 60          |         |
| female           | 14               | 34          |         |

(B)

|            | SELL            | p Value |
|------------|-----------------|---------|
| KD1        |                 |         |
| Cal(−)     | 1.89 ± 0.19     | 0.118   |
| Cal(+)     | 1.78 ± 0.31     |         |
| p Value    |                 |         |
| KD2        |                 |         |
| Cal(−)     | 1.06 ± 0.15     | 0.525   |
| Cal(+)     | 0.94 ± 0.13     |         |
| p Value    |                 |         |
| KD3        |                 |         |
| Cal(−)     | 1.74 ± 0.33     | 0.417   |
| Cal(+)     | 1.20 ± 0.23     |         |
| p Value    |                 |         |
| KD4        |                 |         |
| Cal(−)     | 2.53 ± 0.38     | 0.070   |
| Cal(+)     | 1.55 ± 0.18     |         |
| p Value    |                 |         |

(*p < 0.05).

Table 4. (A) Demographic characteristics of the subjects with Kawasaki disease (KD) for IL10RA mRNA; (B) IL10RA mRNA levels between patients with and without coronary artery lesions (CAL).

(A)

|                  | Febrile Controls | KD          | p Value |
|------------------|------------------|-------------|---------|
|                  | (n = 32)         | (n = 43)    |         |
| Age (years)      | 2.53 ± 0.24      | 2.16 ± 0.29 | 0.055   |
| male             | 20               | 34          |         |
| female           | 12               | 9           | 0.114   |

(B)

|            | IL10RA          | p-Value     |
|------------|-----------------|-------------|
| KD1        |                 |             |
| Cal(−)     | 2.16 ± 0.33     | 0.535       |
| Cal(+)     | 2.17 ± 0.54     |             |
| p-Value    |                 |             |
| KD2        |                 |             |
| Cal(−)     | 1.97 ± 0.30     | 0.530       |
| Cal(+)     | 1.32 ± 0.36     |             |
| p-Value    |                 |             |
| KD3        |                 |             |
| Cal(−)     | 2.41 ± 0.29     | 0.876       |
| Cal(+)     | 2.30 ± 0.54     |             |
| p-Value    |                 |             |

(*p < 0.05).
4. Discussion

To our knowledge, this is the first study to explore the expression of eosinophil subtype markers in KD and the first to track them during the course of KD-related disease. It is critical to determine whether eosinophils are involved during KD. Research on the role of eosinophils will guide new strategies for allergic intervention after KD. We present evidence here that dynamically different eosinophilic involvement exists between KD patients with and without CAL.

The protein produced by the representative gene CD101 of iEOS is immunoglobulin superfamily member 2. As both classically activated macrophages and markers of alternately activated macrophages show increased mRNA expression during the acute phase
of KD, iEOS is also important in KD [33]. Yoon et al. performed experimental studies and showed iEOS with an expression of Toll-like receptor (TLR) 4, and IL10R regulates inflammatory and anti-inflammatory polarization of macrophages, which is very similar to the behavior of macrophages in acute KD [34]. The decrease in IL10RA mRNA after IVIG is consistent with previous reports of decreased ECP after IVIG [8,25]. The results of increased IL10RA between KD and controls as written here support previous findings on diagnostic biomarkers for KD [16]. The enhancement of IgE-mediated mast cell activation by IL-10 is critical for the development of food allergy in mouse models [35]. Lee et al. found significantly elevated IL-10 in KD patients [36]. The alpha subunit of the IL-10 receptor (encoded in the IL10RA gene) is expressed on hematopoietic cells such as T, B, NK, mast, dendritic cells, and eosinophils. Interleukin-10 receptor expression and signaling are downregulated in CD4+ T cells from lupus nephritis patients with higher clinical scores [37]. Eosinophils can promote autoantibody-driven inflammatory processes in anti-neutrophil cytoplasmic antibody-associated vasculitis by increasing the surface expression of activating Fc receptors [38].

RT-PCR validation of SELL showed no difference in SELL between febrile controls, which is in line with the literature showing no difference in CD62L expression on polymorphonuclear leukocytes between KD patients and normal subjects [39]. However, single-cell RNA sequencing identified expanded SELL+CD14+CD16- monocytes, which were poorly differentiated and associated with neutrophil activation in KD [40]. The discrepancy between SELL’s gene expression array and RT-PCR validation results may be due to the increased number of cases. Among other important and distinct rEOS genes in KD, SERPINB1, which encodes SerpinB1 belonging to the serine protease inhibitor family, functions as a signature gene for maintaining neutrophil survival and Th17 cells [41]. The elevation of SERPINB1 is consistent with a Th17/Treg imbalance in KD [42]. SerpinB1 is involved in inhibiting acute oxidative stress and attenuating endothelial cell apoptosis [43]. L-selectin is the smallest angioselectin encoded by SELL and is also known as CD62L. Recent evidence suggests that L-selectin overexpression in multiple tumor-infiltrating immune cells mitigates tumor growth and correlates with favorable survival outcomes in breast cancer [44]. The expression of SELL and SERPINE1 in KD demonstrates the role of rEOS in regulating immunity overactivated by KD immunity.

Among the many topics to be explored in future research, some important ones can be listed as follows. Prospective cohort studies that allow multiple testing are required to characterize the subtype properties of eosinophils and their possible roles in the pathogenesis of inflammation or anti-inflammatory effects against KD. Future work will hopefully clarify the important concern about the histological proof of eosinophils in situ by mouse models [45]. Neutralizing antibodies directed against IL-5 (α–IL-5) treatment did not impact the numbers of rEOS but substantially inhibited the recruitment of iEOS in the mice’s lungs. Anti-IL5 for KD patients with an iEOS phenotype or in KD patients with prognostic biomarkers of future allergic diseases would be considered [46]. An area of future research that should be considered is prognostic and therapeutic biomarkers of long-term allergic complications, the influence on acute treatment, and the strategy for preventing them.

5. Concluding Remarks

The conclusions drawn from the observations were mainly based on the following investigations: KD patients without CAL had lower SELL mRNA levels than patients with CAL, and KD patients with lower IL10RA mRNA levels were more likely to develop CAL. Evidence from various sources suggests that eosinophil subtypes may play a role in KD. The eosinophilic subtype is far from being functionally understood and characterized. The assessment of how inflammatory responses are modulated may contribute to the interpretation and confirmation of eosinophil subtypes associated with KD.
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Abbreviations

CAL, coronary artery lesions; ECP, eosinophil cationic protein; iEOS, inflammatory eosinophils; IgE, immunoglobulin; IL, interleukin; IVIG, intravenous immunoglobulin; KD, Kawasaki disease; LTB4, Leukotriene B4; M1, classically activated macrophages; M2, alternatively activated macrophages; rEOS, lung-resident eosinophils; RT-PCR, quantitative reverse transcriptase-polymerase chain reaction; SELL, Selectin L.

References

1. Lei, W.T.; Chang, L.S.; Zeng, B.Y.; Tu, Y.K.; Uehara, R.; Matsuoka, Y.J.; Su, K.P.; Lee, P.C.; Cavalcante, J.L.; Stubbs, B.; et al. Pharmacologic interventions for Kawasaki disease in children: A network meta-analysis of 56 randomized controlled trials. *EBioMedicine* 2022, 78, 103946. [CrossRef] [PubMed]

2. Webster, R.J.; Carter, K.W.; Warrington, N.M.; Loh, A.M.; Zaloumis, S.; Kuipers, T.W.; Palmer, L.J.; Burgner, D.P. Hospitalisation with infection, asthma and allergy in Kawasaki disease patients and their families: Genealogical analysis using linked population data. *PLoS ONE* 2011, 6, e28004. [CrossRef] [PubMed]

3. Lei, W.-T.; Hsu, C.-W.; Chen, P.-C.; Tseng, P.-T.; Kuo, H.-C.; Guo, M.M.-H.; Tu, Y.-K.; Lin, P.-Y.; Kao, Y.-H.; Chang, L.-S. Increased Risk of Asthma and Allergic Rhinitis in Patients with a Past History of Kawasaki Disease: A Systematic Review and Meta-Analyses. *Front. Pediatrics* 2021, 9, 746856. [CrossRef] [PubMed]

4. Huang, P.Y.; Huang, Y.H.; Guo, M.M.; Chang, L.S.; Kuo, H.C. Kawasaki Disease and Allergic Diseases. *Front. Pediatrics* 2020, 8, 614386. [CrossRef]

5. Chang, L.S.; Chen, Y.J.; Huang, P.Y.; Chen, K.D.; Lo, M.H.; Huang, Y.H.; Guo, M.M.; Kuo, H.C. Significantly Lower Immunoglobulin M Levels 6 Months after Disease Onset in Patients with Kawasaki Disease with Coronary Artery Lesions. *J. Am. Heart Assoc.* 2021, 10, e020505. [CrossRef]

6. Chang, L.S.; Guo, M.M.; Yan, J.H.; Huang, Y.H.; Lo, M.H.; Kuo, H.C. Low FCMR mRNA expression in leukocytes of patients with Kawasaki disease six months after disease onset. *Pediatric Allergy Immunol. Off. Publ. Eur. Soc. Pediatric Allergy Immunol*. 2020, 31, 554–559. [CrossRef]

7. Kuo, H.C.; Chang, W.C.; Yang, K.D.; Yu, H.R.; Wang, C.L.; Ho, S.C.; Yang, C.Y. Kawasaki disease and subsequent risk of allergic diseases: A population-based matched cohort study. *BMC Pediatrics* 2013, 13, 38. [CrossRef]

8. Kuo, H.C.; Wang, C.L.; Liang, C.D.; Yu, H.R.; Huang, C.F.; Wang, L.; Hwang, K.P.; Yang, K.D. Association of lower eosinophil-related T helper 2 (Th2) cytokines with coronary artery lesions in Kawasaki disease. *Pediatric Allergy Immunol. Off. Publ. Eur. Soc. Pediatric Allergy Immunol*. 2009, 20, 266–272. [CrossRef]

9. Kuo, H.C.; Yang, K.D.; Liang, C.D.; Song, C.N.; Yu, H.R.; Wang, L.; Wang, C.L. The relationship of eosinophilia to intravenous immunoglobulin treatment failure in Kawasaki disease. *Pediatric Allergy Immunol. Off. Publ. Eur. Soc. Pediatric Allergy Immunol*. 2007, 18, 354–359. [CrossRef]

10. Kim, J.J.; Kim, H.J.; Yu, J.J.; Yun, S.W.; Lee, K.Y.; Yoon, K.L.; Kil, H.R.; Kim, G.B.; Han, M.K.; Song, M.S.; et al. IgA Levels Are Associated with Coronary Artery Lesions in Kawasaki Disease. *Korean Circ. J.* 2021, 51, 267–278. [CrossRef]

11. Berek, C. Eosinophils: Important players in humoral immunity. *Clin. Exp. Immunol. 2016*, 183, 57–64. [CrossRef] [PubMed]
37. Cui, H.D.; Qi, Z.M.; Yang, L.L.; Qi, L.; Zhang, N.; Zhang, X.L.; Du, S.Y.; Jiang, Y. Interleukin-10 receptor expression and signalling were down-regulated in CD4+ T cells of lupus nephritis patients. *Clin. Exp. Immunol.* 2011, 165, 163–171. [CrossRef]

38. Hellmark, T.; Ohlsson, S.; Pettersson, Å.; Hansson, M.; Johansson, Å.C.M. Eosinophils in anti-neutrophil cytoplasmic antibody associated vasculitis. *BMC Rheumatol.* 2019, 3, 9. [CrossRef]

39. Kobayashi, T.; Kimura, H.; Okada, Y.; Inoue, Y.; Kobayashi, T.; Shinohara, M.; Morikawa, A. Increased CD11b expression on polymorphonuclear leucocytes and cytokine profiles in patients with Kawasaki disease. *Clin. Exp. Immunol.* 2007, 148, 112–118. [CrossRef]

40. Geng, Z.; Tao, Y.; Zheng, F.; Wu, L.; Wang, Y.; Wang, Y.; Sun, Y.; Fu, S.; Wang, W.; Xie, C.; et al. Altered Monocyte Subsets in Kawasaki Disease Revealed by Single-cell RNA-Sequencing. *J. Inflamm. Res.* 2021, 14, 885–896. [CrossRef]

41. Hou, L.; Yuki, K. SerpinB1 expression in Th17 cells depends on hypoxia-inducible factor 1-alpha. *Int. Immunopharmacol.* 2020, 87, 106826. [CrossRef] [PubMed]

42. Jia, S.; Li, C.; Wang, G.; Yang, J.; Zu, Y. The T helper type 17/regulatory T cell imbalance in patients with acute Kawasaki disease. *Clin. Exp. Immunol.* 2010, 162, 131–137. [CrossRef] [PubMed]

43. Liang, X.; Su, Y.; Huo, Y. Forkhead box protein O1 (FoxO1)/SERPINB1 ameliorates ROS production in diabetic nephropathy. *Food Sci. Nutr.* 2021, 9, 44–51. [CrossRef]

44. Kumari, S.; Arora, M.; Singh, J.; Chauhan, S.S.; Kumar, S.; Chopra, A. L-Selectin expression is associated with inflammatory microenvironment and favourable prognosis in breast cancer. *3 Biotech* 2021, 11, 38. [CrossRef] [PubMed]

45. Noval Rivas, M.; Arditi, M. Kawasaki disease: Pathophysiology and insights from mouse models. *Nat. Rev. Rheumatol.* 2020, 16, 391–405. [CrossRef] [PubMed]

46. Lombardi, C.; Berti, A.; Cottini, M. The emerging roles of eosinophils: Implications for the targeted treatment of eosinophilic-associated inflammatory conditions. *Curr. Res. Immunol.* 2022, 3, 42–53. [CrossRef]