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Transcript abundance patterns in Kawasaki disease patients with IVIG resistance

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Abstract

Intravenous immunoglobulin (IVIG)-resistant Kawasaki disease (KD) patients comprise at least 20% of treated patients and are at higher risk of coronary artery abnormalities. If identified early in the course of the disease, such patients may benefit from additional anti-inflammatory therapy. The aim of this study was to compare the transcript abundance between IVIG-resistant and – responsive KD patients to identify biomarkers that might differentiate between these two groups and to generate new targets for therapies in IVIG-resistant KD patients. We compared the transcript abundance profiles of whole blood RNA on Agilent arrays from acute and convalescent KD subjects and age-similar, healthy controls. KD subjects were stratified as IVIG-resistant or – responsive based on response to initial IVIG therapy. Transcript abundance was higher for IL-1 pathway genes (IL-1 receptor, interleukin receptor associated kinase, p38 mitogen-activated protein kinase), and *MMP-8*. These findings point to candidate biomarkers that may predict IVIG-resistance in acute KD patients. The results also underscore the importance of the IL-1 pathway as a mediator of inflammation in KD and suggest that IL-1 or its receptor may be reasonable targets for therapy, particularly for IVIG-resistant patients.

Keywords

Kawasaki disease; gene expression; intravenous immunoglobulin

1. Introduction

Transcript abundance can be a window into the host response to an inflammatory stimulus. From previous transcript abundance studies of Kawasaki disease (KD), the leading cause of

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acquired heart disease in children, we have learned of the dynamic nature of the host response in the first week following onset of fever. Investigators have focused on groups of genes in pathways implicated in inflammation, endothelial cell injury, and cardiomyocyte injury [1-5].

Treatment with intravenous immunoglobulin (IVIG) remains the mainstay of therapy for KD and reduces the rate of coronary aneurysm formation from 25% to 3-5% [6]. Resistance to IVIG therapy appears to be increasing with reported resistance rates of 20-30% in Japan and the U.S. [7-9]. IVIG-resistance is a major risk factor for development of aneurysms and early identification of patients at risk would allow timely administration of additional anti-inflammatory therapies [8,10]. Although clinical scoring systems to identify potential IVIG-resistant KD patients have been validated for Japanese populations, such scoring systems were insufficiently robust to be clinically useful in an ethnically diverse population from the United States [8,11-14]. Thus, a better understanding of the pathogenetic mechanisms underlying IVIG-resistance is needed.

We compared whole blood transcript levels among acute KD subjects prior to initial IVIG administration, the same subjects during convalescence, and healthy controls. KD subjects were stratified for analysis based on clinical response to IVIG. We performed a sequential, pairwise comparison of transcript abundance. From this analysis, we identified novel biological pathways that characterized acute KD and transcript signatures associated with IVIG-resistance. This analysis highlights the role of cytokine networks involving IL-1 and matrix metalloproteinase-8 (MMP-8) and suggests a role for IL-1 antagonists in the treatment of IVIG-resistant KD.

2. Subjects and Methods

2.1 Study populations and sample collection

KD patients (n=20) and healthy controls (n=9) were enrolled at Rady Children's Hospital San Diego after obtaining parental informed consent. KD subjects with a spectrum of illness days, ethnicities, and disease severity admitted to Rady Children's Hospital San Diego between 2003 and 2006 were chosen based on availability of acute and convalescent samples. The human subjects' protocol was reviewed and approved by the University of California, San Diego, Institutional Review Board. All patients diagnosed with KD had fever for at least 4 days and met at least 4 of 5 clinical criteria for KD (rash, conjunctival injection, cervical lymphadenopathy, oral mucosal changes, and changes in the extremities) or 3 of 5 criteria and coronary artery abnormalities documented on echocardiogram[15]. Blood was obtained at the time of diagnosis prior to initial IVIG treatment (acute phase) and again approximately 1 month following the resolution of the acute illness after the erythrocyte sedimentation rate (ESR) and platelet count had returned to normal (convalescent phase), from all but one subject from whom only an acute sample was available. Whole blood was collected in PAXgene tubes and total RNA was isolated as previously described [1]. Healthy age-similar controls were children <5 years of age undergoing elective minor orthopedic surgical procedures. Whole blood was collected in PAXgene tubes prior to general anesthesia.

Demographic and clinical characteristics of study subjects are presented in Table 1. Clinical laboratory evaluations included complete blood count and differential, C-reactive protein level, and ESR. Sex, age, illness day at sample collection (illness day 1= 1st calendar day of fever), response to IVIG, and echocardiographic measurement of coronary arteries were recorded for all KD patients. Measurements of the internal diameter of the right and left anterior descending coronary arteries were normalized based on body surface area and expressed as a z score (standard deviation units from the mean) [16,17]. Arteries were classified as normal, dilated or aneurysmal (z score <2.5, ≥2.5 to <4.0, or ≥4.0, respectively). KD subjects were IVIG-resistant (n=8) if they had persistent or recrudescent fever at least 36 hours after the end of

their IVIG infusion. IVIG-resistance was treated with either infliximab at 5 mg/kg IV (n=5) or a second dose of IVIG at 2 g/kg IV (n=3).

2.2 Agilent Whole Human Genome Oligo Microarray Experiment

Aminoallyl cRNA was produced from 1 µg of total RNA using Fluorescent Linear Amplification Kit (Agilent Technologies) and aminoallyl-UTP. Cyanine (Cy) 3 or Cy5 was chemically conjugated to aminoallyl cRNA using Cy3 or Cy5 NHS ester (GE Life Sciences). All sample RNAs were labeled with Cy3 and compared to a human blood common reference RNA labeled with Cy5. Cy3 and Cy5 labeled cRNAs were hybridized to the Agilent G4112F Human Whole Genome Oligo Arrays (design number 14850) per the manufacturer's instructions except for the following modification: cRNAs containing 300 pmol of Cy3 and 150 pmol of Cy5 were used and the hybridization was done at 70°C. Hybridized microarrays were washed according to the manufacturer's protocol and scanned on an Agilent Microarray Scanner. Raw data were extracted from scanned array images using Agilent Feature Extraction 9.5.1.1. Ratios were calculated after subtracting the average of negative control signals (background) and normalization by non-linear Lowess normalization.

2.3 Quantitative RT-PCR

Whole blood mRNA levels for *MMP-8* were measured in 8 IVIG-resistant and 12 IVIG-responsive KD subjects using TaqMan Gene Expression assay (Hs01029057_m1, Applied Biosystem). Relative abundance of the target transcripts was normalized to the expression level of the housekeeping gene, TATA box binding protein-associated factor RNA polymerase I B (*TAF1B*) (Hs01057259_m1, Applied Biosystem) as previously described [1].

2.4 MMP-8 Serum Levels

We measured acute and convalescent serum levels of MMP-8 in an independent cohort of 26 consecutive, unselected KD subjects. The cohort characteristics were as follows: median age 2.9 years (range 0.37 to 11 years); 69% male; 19% Caucasian; 57% IVIG responders; 65% normal coronaries; 19% dilated coronaries; 15% aneurysms. MMP-8 levels were measured using the Fluorokine MAP Assay (R&D Systems) as per manufacturer's protocol. MMP-8 concentrations were calculated using Bio-Plex Manager 5.0 software with a 5 parameter curve fitting algorithm applied for standard curve calculations.

2.5 Statistical analysis

We first identified the transcripts that were differentially abundant between all KD subjects and healthy controls (Figure 1). Then we selected from those transcripts the ones that were also differentially abundant between acute and convalescent KD subjects. Lastly, we identified the KD-specific transcripts that were also differentially abundant between IVIG-responsive and -resistant subjects.

Genes whose average raw probe intensities were lower than 100 across the samples analyzed were removed from further analysis. To identify genes with differentially abundant transcripts between the two groups of samples, a filter of average fold change >1.5 was applied. If the same probe sequence was on more than one spot on the microarray chip, the signal intensity for that probe was averaged. Comparisons between two groups for continuous variables were made with a Student's t-test or a Mann Whitney *U* test for parametric and non-parametric data, respectively. Non-parametric microarray data were log-transformed and analyzed by Student t-test. If more than one probe existed for a given gene, the average of the *p* values of the log-transformed data was reported. Results were considered statistically significant if *p* < 0.05. False Discovery Rate (FDR) *q* value was calculated using the formula ($p * n / i$) where *p* is the *p* value of the Student test of the probe, *n* is the number of total Student *t* tests, and *i* is the

ascending sorted rank of the p value. Pearson's correlation and the correlation coefficients were calculated using Prism to evaluate the relationship between microarray and RT-PCR results. The overlap genes of two lists were obtained by using GeneSpring GX 7.3 software by Agilent® Technologies (www.agilent.com.) Hypergeometric probability tests were performed with R (http://cran.r-project.org/). Genes associated with probes of interest were identified and assigned to pathways by Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com).

3. Results

3.1 Increased MMP-8 transcript abundance in KD versus healthy control subjects

Microarray data were compared in 3 different pairwise analyses to identify sets of probes with > 1.5-fold differences between acute KD subjects and healthy controls, between acute and convalescent KD subjects, and between IVIG-resistant and –responsive acute KD subjects (Table 2). Of the 4,799 probes that were less abundant during the acute phase of KD compared with healthy controls, 2,200 probes were also more abundant during the convalescent phase of KD and thus represented disease-specific transcripts that were differentially abundant between the acute and convalescent phase (Figure 2). Of the 6,875 probes that were more abundant in acute KD subjects vs. healthy controls, 2,575 probes were more abundant in acute KD subjects vs. both convalescent KD subjects and healthy controls. Of the 4,775 acute-phase, disease-specific probes differentially expressed in acute KD subjects compared with convalescent KD subjects and healthy controls, we selected for analysis the 200 probes that had the largest fold difference in transcript abundance between acute KD subjects and healthy controls (maximum q value 3.23%, Supplemental Table 1). Of these probes, 173 were more abundant in acute KD subjects than in healthy controls and 27 were less abundant. Ingenuity Network Pathway Analysis revealed that the genes associated with these 200 probes were in Inflammatory Disease and Immunological Disease pathways (ranges of p values 2.96×10^{-16} to 9.52×10^{-3} and 6.22×10^{-15} to 9.52×10^{-3} , respectively) and included *MMP-8* as the most abundant transcript and nuclear factor kappa B (NF- κ B) signaling proteins. Also included were S100 proteins (S100A8, S100A9 and S100A12) and carcinoembryonic antigen-related cell adhesion molecules (CEACAM 1 and CEACAM 8) that had been identified in previous microarray analyses by our group and others as being associated with IVIG resistance (Figure 3) [1,2, 18-20]. Ingenuity Canonical Pathway Analysis found that the most shared pathway for these top 200 probe-related genes was the Toll-like receptor signaling pathway with a p value of 1.18×10^{-03} (Figure 4) [21].

3.2 Transcript abundance profiles reveal a role for the IL-1 signaling pathway in IVIG-resistant KD patients

Comparison between IVIG-responsive and –resistant KD subjects revealed 3,372 probes that were differentially expressed between these two groups, with 1,457 probes more abundant and 1,915 probes less abundant in IVIG-resistant subjects (Table 2). We compared the 2,575 disease-specific probes more abundant in acute KD than in healthy controls and convalescent KD with the 1,457 probes more abundant in IVIG-resistant subjects versus IVIG-responsive subjects (Figure 5). Among the resulting 356 probes, in order of abundance, were *MMP-8*, *IL1 receptor 2*, *S100A8*, *interleukin-1 receptor-associated kinase*, *S100A9*, and *CEACAM1* ($p < 2.2 \times 10^{-16}$; maximum q value 3.81%, Supplemental Table 2). The Ingenuity Canonical Pathway Analysis found that these 356 probes were associated with a broad range of genes in the IL-1 signaling pathway that encoded proteins expressed at the cell surface, in the cytoplasm, and in the nucleus (Figure 6) [22-25].

We also tested the reciprocal situation with transcripts that were less abundant in acute KD and more abundant in IVIG resistance and transcripts that were more abundant in acute KD

and less abundant in IVIG resistance. There was no significant overlap between these probe sets. Therefore, acute KD subjects and IVIG-resistant subjects shared the same pattern of gene expression with the IVIG-resistant subjects having higher transcript abundance of disease-specific genes.

3.3 Transcript abundance and serum levels of MMP-8

The probe for MMP-8 had the highest fold difference in acute KD compared to both convalescent KD and healthy controls, as well as in IVIG-resistant KD subjects compared to IVIG-responsive subjects. RT-PCR for MMP-8 transcripts from IVIG-resistant and IVIG-responsive subjects showed a strong correlation with the microarray results (correlation coefficient $r=0.91$; $p<0.0001$, 95% CI 0.79 to 0.97), thus confirming elevated levels of MMP-8 mRNA in IVIG-resistant subjects (Figure 7).

MMP-8 serum levels were measured in an independent cohort of 26 paired acute and convalescent KD subjects. A statistically significant difference in median levels of MMP-8 was seen in this comparison with a median of 47,672 pg/ml and 10,371 pg/ml in acute and convalescent KD serum samples, respectively. ($p<0.0001$) (Figure 8). The MMP-8 serum levels were also measured between IVIG-resistant and -responsive KD subjects. No difference was detected between the median MMP-8 levels of these two groups, although power to detect a difference was limited by the small sample size.

3.4 Abundance of S100 proteins and CEACAM1 transcripts in IVIG-resistant acute KD

Transcripts of genes reported to be differentially expressed in KD were compared across acute and convalescent IVIG-resistant and IVIG-responsive subjects as well as healthy controls. Signal intensity was low for *CEACAM1*, *S100A12*, *S100A8*, *S100A9* and *C1Q* in convalescent IVIG-resistant and IVIG-responsive KD subjects and healthy controls compared to acute KD subjects. During the acute phase, IVIG-responsive and IVIG-resistant KD subjects had similar elevations in *C1Q* and *S100A12* probe intensity levels, which decreased in the convalescent phase (data not shown). Probe intensity for *CEACAM1*, *S100A8*, and *S100A9* was higher in IVIG-resistant subjects compared to IVIG-responsive subjects ($p=0.027$, 0.027 , and 0.040 respectively) (Supplemental Figure 1).

4. Discussion

We analyzed whole blood transcript abundance in acute and convalescent KD subjects and healthy pediatric controls. In an Ingenuity Pathway Analysis, the 200 highest intensity probes associated with acute KD were from genes in pathways for inflammatory and immunological diseases. Toll-like receptor signaling was the most significant canonical pathway of these top 200 probes. Subjects with acute KD were further subdivided based on response to therapy. Analysis of the 356 probes that were elevated both between acute and convalescent KD subjects and between IVIG-responsive and -resistant subjects demonstrated the importance of the IL-1 signaling pathway. In addition, probes for *MMP-8* had the highest intensity and *MMP-8* was the most differentially abundant transcript when comparing IVIG-responsive and -resistant KD subjects. This finding was confirmed by RT-PCR. This is the first microarray study to demonstrate that the IL-1 pathway and *MMP-8* are important in KD pathogenesis, and, therefore, potential targets for further study and therapeutic intervention.

While increased transcript abundance for other matrix metalloproteinases, including *MMP-9*, has been found by microarray analysis of whole blood from acute KD subjects, this is the first time that increased transcript abundance of *MMP-8* has been reported. This is important because *MMP-8*, also known as neutrophil collagenase, cleaves type 1 collagen in the extracellular matrix. Levels of *MMP-8* are elevated in ruptured infarct tissue as compared to

control myocardial infarction tissue, thus demonstrating the role of MMP-8 in myocardial destruction which may also be a factor in KD [26]. The previous microarrays used by our group did not have MMP-8 probes on the chip (Lymphochip, Stanford University) [1,27]. The high levels of MMP-8 expression in KD [28,29] subjects suggest that this collagenase may be a potential therapeutic target. The antibiotic, doxycycline, has been shown to inhibit abdominal aortic aneurysm formation in animals models and in limited human clinical trials by inhibiting matrix metalloproteinases [30-33]. A recent randomized, dose escalation study in adults with abdominal aortic aneurysms demonstrated reduced MMP-8 and MMP-9 levels in plasma as well as reduced neutrophil infiltration into the aortic wall in subjects treated with doxycycline compared to control subjects prior to surgical resection of the aneurysm [34]. These data suggest that doxycycline may be an effective adjuvant therapy for KD patients.

The increased transcript abundance of several genes in the IL-1 pathway in IVIG-resistant subjects is a novel finding and suggests that IL-1 may also be a therapeutic target in KD. Higher transcript abundance for IL-1 pathway genes was also found in a comparison of acute KD subjects with febrile control subjects with documented adenovirus infection [27]. A variety of biologic agents target the IL-1 pathway and include anakinra, an IL-1 receptor antagonist, the fusion protein rilonacept (Arcalyst®), and the monoclonal antibody canakinumab (Ilaris®) [28,35,36]. Targeted anti-IL-1 therapy may be a promising adjuvant therapy for acute KD. Given the acute, self-limited nature of KD, only a single dose would likely be required, which would circumvent the side effects associated with chronic use.

NF- κ B is a critical transcription factor for genes that encode proinflammatory cytokines, so it was not surprising that several of the genes from the list of the top 200 probes with differential intensity between acute and convalescent KD subjects are members of this signaling pathway. NF- κ B signaling has been shown to decrease in CD14+ monocytes/ macrophages from IVIG-responsive KD subjects after IVIG infusion [37]. The NF- κ B signaling cascade is activated by several proinflammatory cytokines including tumor necrosis factor (TNF)- α which is a target in a current clinical trial for treatment of acute KD [38].

The rate of IVIG-resistance is increasing in Japan and the U.S. [7-9]. Since IVIG-resistance is associated with an increased rate of coronary artery aneurysms, it would be important to identify IVIG-resistant KD patients before initiation of therapy as they may benefit from additional anti-inflammatory treatment [8]. Our microarray analysis identified several genes that may be important both in predicting IVIG resistance and in defining disease-specific therapeutic targets for IVIG-resistant KD patients. Increased transcript abundance of the neutrophil-associated calcium binding proteins S100A8 and A9 in IVIG-resistant KD subjects confirms the role of activated neutrophils in the acute phase of KD. S100A8 and A9 have also been shown to regulate adhesion of neutrophils and monocytes to endothelial cells, a process that is likely important in the vasculitis of KD [20]. Given the role of the S100A8/A9 heterodimer in activating interleukin-1 receptor associated kinase-1 and NF- κ B, which induce TNF α , it is not surprising that increased transcript abundance of these genes is associated with more inflammation and IVIG-resistance [39]. Increased transcript abundance of S100A9 was reported in acute vs. convalescent KD peripheral blood mononuclear cells [2,3,40]. The increased mRNA levels of several S100 proteins, including S100A8 and A9, have been previously confirmed by RT-PCR [2,4,41]. Persistent elevation of the plasma S100A8/S100A9 heterocomplex in acute KD has been associated with coronary artery aneurysms [2]. Taken together, these data emphasize the role of S100A8 and A9 in KD disease pathogenesis and suggest that these proteins may be useful biomarkers for identifying IVIG-resistant KD patients.

Our study confirms previous work by our group documenting the differential expression of *CEACAM1* in IVIG-resistant vs. -responsive KD patients [1]. The *CEACAM1* protein appears

to have a role in angiogenesis via the vascular endothelial growth factor (VEGF) pathway [42]. Elevated plasma levels of VEGF have been detected in acute KD and immunohistochemistry has demonstrated VEGF in coronary arteries, where it may mediate the prominent neovascularization seen in the arterial wall [43,44]. Increased *CEACAM1* expression has also been associated with delayed neutrophil apoptosis, which suggests that apoptosis may be an important pathway for decreasing inflammation in KD [18,45,46]. Therefore, measuring concentrations of the alternatively spliced variants of *CEACAM1* in plasma may reveal it to be a useful biomarker in predicting IVIG-resistance.

We recognize several limitations to our study. The cohort size was small for both our KD subjects and healthy controls and the small number of subjects with coronary artery abnormalities limited our power to detect a difference between groups. Transcript abundance patterns in whole blood may not accurately reflect gene expression patterns in the tissues where the pathologic changes associated with this vasculitis occur.

Conclusions

The increasing rate of IVIG-resistance in KD patients lead us to seek clues from whole blood transcript abundance patterns to identify biomarkers of IVIG-resistance and new targets for therapies in IVIG-resistant patients. Higher *MMP-8*, *S100A8*, *S100A9*, and *CEACAM1* transcript levels were associated with IVIG-resistance, suggesting that these may be potential biomarkers for identifying these KD patients who are at highest risk for coronary artery aneurysms and might benefit from additional anti-inflammatory therapy. The novel finding that the IL-1 pathway and MMP-8 are important in acute KD suggests these as potential therapeutic targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

KD	Kawasaki disease
IVIG	intravenous immunoglobulin
ESR	erythrocyte sedimentation rate
IL	interleukin
MMP	matrix metalloproteinase
CEACAM	carcinoembryonic antigen-related cell adhesion
NF-κB	Nuclear factor kappa B
VEGF	vascular endothelial growth factor

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Figure 1.
Flow diagram of the groups compared for transcript abundance

Figure 2. Venn diagram of disease-specific transcripts differentially abundant in acute KD subjects (n=20) compared to healthy controls (n=9) and convalescent KD subjects (n=20)

A. Overlap of probes that were less abundant in acute KD subjects and more abundant in both healthy controls and convalescent KD subjects. B. Overlap of probes that were more abundant in acute KD subjects and less abundant in both healthy controls and convalescent KD subjects.

Figure 3. Ingenuity Network Pathway Analysis of MMP-8, S100 proteins (S100A8, S100A9 and S100A12), and carcinoembryonic antigen-related cell adhesion molecules (CEACAM 1 and CEACAM 8)

A grey shaded molecule refers to a probe that was more abundant in acute KD subjects vs. both convalescent KD subjects and healthy control.

Network Shapes

Cytokine
Growth Factor
Chemical / Drug/ Toxicant
Enzyme
G-protein Coupled Receptor
Ion Channel
Kinase
Ligand-dependent Nuclear Receptor
Peptidase
Phosphatase
Transcription Regulator
Translation Regulator
Transmembrane Receptor
Transporter
microRNA
Complex / Group
Other

Path Designer Shapes

Cytokine / Growth Factor
Drug
Chemical / Toxicant
Enzyme
G-protein Coupled Receptor
Ion Channel
Kinase
Ligand-dependent Nuclear Receptor
Peptidase
Phosphatase
Transcription Regulator
Translation Regulator
Transmembrane Receptor
Transporter
microRNA
Complex / Group
Other

Relationships

—————
direct interaction

- - - - -
indirect interaction

Note: "Acts on" and "inhibits" edges may also include a binding event.

Figure 4. Ingenuity Canonical Pathway Analysis of the genes associated with the top 200 probes that are differentially abundant between acute KD subjects and both convalescent KD subjects and healthy control

A grey shaded molecule refers to a probe that was more abundant in acute KD subjects vs. both convalescent KD subjects and healthy control.

Figure 5. Venn diagram of overlap between disease-specific transcripts differentially abundant in acute KD (n=20) and IVIG-resistant KD subjects (n=8)

Overlap of probes that were more abundant in acute KD and IVIG-resistant KD subjects than in IVIG-responsive KD subjects.

p38 MAPK Signaling

Figure 6. Ingenuity Canonical Pathway Analysis of the genes associated with the 356 probes that were increased in both acute KD and IVIG-resistant KD subjects

A grey shaded molecule refers to a probe that was more abundant in acute KD subjects vs. both convalescent KD subjects and healthy control as well as more abundant in IVIG-resistant compared to IVIG-responsive KD subjects.

Figure 7. Comparison of MMP-8 relative transcript abundance levels by RT PCR in IVIG-resistant and IVIG-responsive KD subjects

Box plots demonstrate the median, 25th and 75th percentiles. Whiskers identify the 5th and 95th percentiles.

Figure 8. Comparison of MMP-8 serum levels in an independent cohort of acute and convalescent KD subjects

Box plots demonstrate the median, 25th and 75th percentiles. Whiskers identify the 5th and 95th percentiles.

Table 1

Demographic and clinical characteristics of study subjects.

	KD IVIG-Responsive (n=12)	KD IVIG-Resistant (n=8)	Healthy Controls (n=9)
Age at Onset, yr	3.29 (0.33-9)	1.85 (0.33-4.92)	1.65 (0.39-4.47)
Male, n (%)	5 (42)	5 (63)	3 (33)
Illness Day at sample collection	6 (4-10)	5 (4-7)	NA
Ethnicity/Race, n (% of total)			
Asian	1 (8.3)	0	0
Caucasian	4 (33)	2 (25)	3 (33)
Hispanic	3 (25)	4 (50)	3 (33)
Mixed	4 (33)	2 (25)	3 (33)
Coronary Artery	8 Normal	3 Normal	NA
Status ^a	4 Dilated	3 Dilated 2 Aneurysms	
White blood cells × 10 ³ /mm ³	13.3 (5.9-23)	13.0 (7.6-19.2)	NA
Neutrophils, %	57 (22-76)	54 (28-73)	NA
Bands, %	8 (0-28)	17 (0-50)	NA
Absolute	8303	9888	NA
Neutrophil Count	(4818-16269)	(4302-14976)	
Lymphocytes, %	17 (5-70)	18 (2-34)	NA
Monocytes, %	4.5 (0-17)	5 (2-10)	NA
zHemoglobin ^b	-0.73 (-4.33-1)	-1.40 (-5.83-0)	NA
Erythrocyte	59.5	63.5	NA
Sedimentation	(27-140)	(40-140)	
Rate, mm/h			
C Reactive	5.5	18	NA
Protein, mg/dL	(0.3-18.4)	(5.1-44.2)	
Platelets × 10 ³ /mm ³	404 (89-541)	394 (85-516)	NA
Alanine	69	12.5	NA
Aminotransferase, U/L	(6-725)	(24-161)	
Gamma Glutamyl	64	109.5	NA
Transferase, U/L	(9-220)	(6-216)	

All data presented as median (range).

^a Only significant difference between IVIG-responsive and -resistant KD subjects, $p=0.04$ chi square test^b zHemoglobin, standard deviation units from the mean based on age-adjusted normal values [44] NA= not available

Pairwise comparisons between healthy controls and acute and convalescent Kawasaki disease subjects who were either IVIG-resistant or IVIG-responsive.

Table 2

Pairwise comparisons*	Healthy controls		KD IVIG-resistant subjects		KD IVIG-responsive subjects		Number of Probes	
	(n=9)	Acute (n=8)	Convalescent (n=8)	Acute (n=12)	Convalescent (n=11)	More abundant in all A vs. all B	More abundant in all B vs. all A	
I	A	B1		B2		4799	6875	
II		A1	B1	A2	B2	3123	2894	
III		A		B		1457	1915	

* Comparisons I and III were analyzed by independent group t tests; Comparison II was analyzed by a dependent group t test