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Journal

PloS one, 11(12)

ISSN

1932-6203

Authors

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Publication Date

2016

DOI

10.1371/journal.pone.0167434

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RESEARCH ARTICLE

Unique Molecular Patterns Uncovered in Kawasaki Disease Patients with Elevated Serum Gamma Glutamyl Transferase Levels: Implications for Intravenous Immunoglobulin Responsiveness

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Citation: Wang Y, Li Z, Hu G, Hao S, Deng X, Huang M, et al. (2016) Unique Molecular Patterns Uncovered in Kawasaki Disease Patients with Elevated Serum Gamma Glutamyl Transferase Levels: Implications for Intravenous Immunoglobulin Responsiveness. PLoS ONE 11 (12): e0167434. doi:10.1371/journal.pone.0167434

Editor: Jagadeesh Bayry, Institut National de la Santeet de la Recherche Medicale (INSERM), FRANCE

Received: July 7, 2016

Accepted: November 14, 2016

Published: December 21, 2016

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Data Availability Statement: This Stanford-led international collaborative effort for Cohort I, II, III, and IV was approved by the Stanford University IRB to perform data science on these datasets due to nature of the patient electronic medical records of collaborative clinical organizations. Due to these ethical restrictions, these data are available upon request. Requests for the data should be directed to Drs. Burns and Lee as following: Clinical

Abstract

Background

Resistance to intravenous immunoglobulin (IVIG) occurs in 10–20% of patients with Kawasaki disease (KD). The risk of resistance is about two-fold higher in patients with elevated gamma glutamyl transferase (GGT) levels. We sought to understand the biological mechanisms underlying IVIG resistance in patients with elevated GGT levels.

Method

We explored the association between elevated GGT levels and IVIG-resistance with a cohort of 686 KD patients (Cohort I). Gene expression data from 130 children with acute KD (Cohort II) were analyzed using the R square statistic and false discovery analysis to identify genes that were differentially represented in patients with elevated GGT levels with regard to IVIG responsiveness. Two additional KD cohorts (Cohort III and IV) were used to test the hypothesis that sialylation and GGT may be involved in IVIG resistance through neutrophil apoptosis.

Results

Thirty-six genes were identified that significantly explained the variations of both GGT levels and IVIG responsiveness in KD patients. After Bonferroni correction, significant

datasets of Cohort I (from November 1989 to August 2014), II (from February 2000 to January 2009), III (from March 2009 to August 2012): Jane C. Burns, MD; jcburns@ucsd.edu, University of California, San Diego (UCSD); Clinical dataset of Cohort IV (from January 2008 to December 2013): JungHwa Lee, MD; leejmd@choi.com. All the gene expression data from Cohort II are available at the GEO public database. The accession number is GSE63881. The normal ranges of GGT and alanine aminotransferase (ALT) are shown in S1A Table and S1B Table respectively, while the normal range of C-reactive protein (CRP) is shown in the footnotes of [S1 File](#).

Funding: This work was supported in part by grants from the American Heart Association to HJC and XBL, Stanford University Spark Program Grant to HJC and XBL, the David Gordon Louis Daniel Foundation to JCB, the Mario Batali Foundation to JCB, the National Institutes of Health, National Heart, Lung, Blood Institute HL69413 to JCB, The Hartwell Foundation to AHT, and The Harold Amos Medical Faculty Development Program/Robert Wood Johnson Foundation to AHT.

Competing Interests: The authors declare that they have no competing interests.

associations with IVIG resistance persisted for 12 out of 36 genes among patients with elevated GGT levels and none among patients with normal GGT levels. With the discovery of *ST6GALNAC3*, a sialyltransferase, as the most differentially expressed gene, we hypothesized that sialylation and GGT are involved in IVIG resistance through neutrophil apoptosis. We then confirmed that in Cohort III and IV there was significantly less reduction in neutrophil count in IVIG non-responders.

Conclusions

Gene expression analyses combining molecular and clinical datasets support the hypotheses that: (1) neutrophil apoptosis induced by IVIG may be a mechanism of action of IVIG in KD; (2) changes in sialylation and GGT level in KD patients may contribute synergistically to IVIG resistance through blocking IVIG-induced neutrophil apoptosis. These findings have implications for understanding the mechanism of action in IVIG resistance, and possibly for development of novel therapeutics.

Introduction

Kawasaki disease (KD) is an acute systemic vasculitis of infants and children, that occurs world-wide [1], but the biology of this condition is not well understood. Coronary artery aneurysms (CAA) occur in 25% of untreated patients, making KD the leading cause of acquired heart disease in children [2, 3]. Therapy for KD includes high-dose aspirin and intravenous immune globulin (IVIG) [4], which reduces the incidence of CAA to 5–7% when given within the first 10 days of illness [5, 6]. However, 10%–20% of patients are resistant to IVIG [7, 8], and are consequently at greater risk of developing CAA, and require additional adjunctive treatments [5, 9]. We [10–12] and others [13–15] have found that serum gamma-glutamyl transferase (GGT) levels are elevated in the acute phase of KD patients and that higher GGT levels are associated with IVIG resistance.

In this study, we explored the association of GGT level and IVIG resistance in KD patients to try to understand the biological mechanism of action underlying these two clinical findings. With our systematic analyses of gene regulation and associated clinical findings and outcomes, we hypothesize: (1) neutrophil apoptosis induced by IVIG plays a pivotal role in IVIG responsiveness; (2) changes in sialylation and GGT levels in acute KD patients may contribute together to IVIG resistance by blocking IVIG-induced neutrophil apoptosis.

Methods

The normal ranges of GGT and alanine aminotransferase (ALT) are shown in Table A in [S1 File](#) and Table B in [S1 File](#) respectively, while the normal range of C-reactive protein (CRP) is shown in the footnotes of Table E in [S1 File](#).

Cohort I: Subjects used to analyze the relationship between the levels of GGT and IVIG resistance

A cohort with 686 subjects with KD (Cohort I) was used to explore the associations between GGT levels and IVIG resistance. All subjects in this cohort were enrolled at Rady Children's Hospital in San Diego after obtaining written parental informed consent and patient assent as appropriate. All subjects were treated with 2 g/kg of IVIG (Gammagard®) over a 10–12 hour

period as per the pharmacy's standard protocol. The study protocol was conducted in accordance with the Declaration of Helsinki and was approved by Institutional Review Boards of UCSD and Stanford University. KD subjects in this study were: a) patients with fever ($\geq 38.0^{\circ}\text{C}$ rectally or orally) for no more than 10 days, plus at least four of the five principal clinical criteria, b) patients meeting fewer criteria but with coronary artery abnormalities (CAA) (Z-score ≥ 2.5 for left anterior descending [LAD] and/or right coronary arteries [RCA]) documented by echocardiogram, and c) patients with fewer than 4 clinical criteria but meeting the American Heart Association (AHA) criteria for incomplete KD by laboratory criteria [4]. KD subjects meeting the inclusion criteria were identified from the database maintained at the UCSD KD Research Center. We obtained prospectively collected demographic and clinical data, the results of laboratory studies prior to IVIG administration, and IVIG-responsiveness. IVIG-resistance was defined as persistent or recurrent fever (rectal or oral temperature $\geq 38.0^{\circ}\text{C}$) at least 36 hours but no longer than 7 days after completion of the initial IVIG infusion (2g/kg) [10]. Subjects in this cohort were either treated with a second course of IVIG or infliximab for IVIG-resistant KD.

Cohort II: Subjects used for gene expression profiling and GGT/IVIG association analysis

We performed gene expression profiles of whole blood obtained from a sub-cohort of 146 KD subjects (Cohort II) as previously described [16]. Of these KD subjects, 130 had complete data on IVIG responsiveness and serum GGT levels available for subsequent analyses; clinical characteristics of this cohort are summarized in Table C in [S1 File](#). The odds ratios of gene expression stratified by IVIG response were calculated using logistic regression with `glm2` in R [17].

For each of the 31,000 genes in the 130 subjects, the explained variations in GGT levels or IVIG responsiveness were estimated using R square of linear regression or the McFadden's pseudo-R square [18, 19] of logistic regression [17], respectively.

Correction for multiple hypothesis testing on the explained variations was performed using the permutation-based false discovery rate (FDR) analytical method [20]. FDRs of GGT- and IVIG-associated variations for each gene were estimated using Monte Carlo simulation. Genes with FDRs less than a threshold of 0.01 were considered true discoveries explaining the variations in GGT levels and IVIG responsiveness. The common genes among true discoveries of both GGT and IVIG were used for the next analysis ([Fig 1](#), Panel 2).

The study population was divided into two sub-cohorts, one with normal and the other with elevated GGT levels, using age-specific reference ranges established in our clinical laboratory (Table A in [S1 File](#)). Logistic regression was performed for each of the 36 common genes with $\text{FDR} < 1\%$, to evaluate its contribution to the risk for IVIG resistance in each sub-cohort. The Bonferroni method was applied to correct for multiple hypothesis testing [21, 22].

Cohorts III and IV: Subjects used to investigate and validate the changes of neutrophils in response to IVIG treatment

Cohort III was derived from the placebo arm of a phase 3, randomized, double-blind, placebo-controlled trial of IVIG with or without infliximab for treatment of KD, which was conducted from March 2009 to August 2012 in two children's hospitals in the USA [23]. Cohort III included 95 KD patients of the 98 KD patients from the placebo arm, and was used to investigate changes of neutrophils in response to IVIG treatment. The 3 excluded placebo subjects had missing data for IVIG response ($n = 1$) or absolute neutrophil count ($n = 2$). The study protocol was reviewed and approved by the Institutional Review Boards at the University of California San Diego's and Nationwide Children's Hospital. Written informed consent was

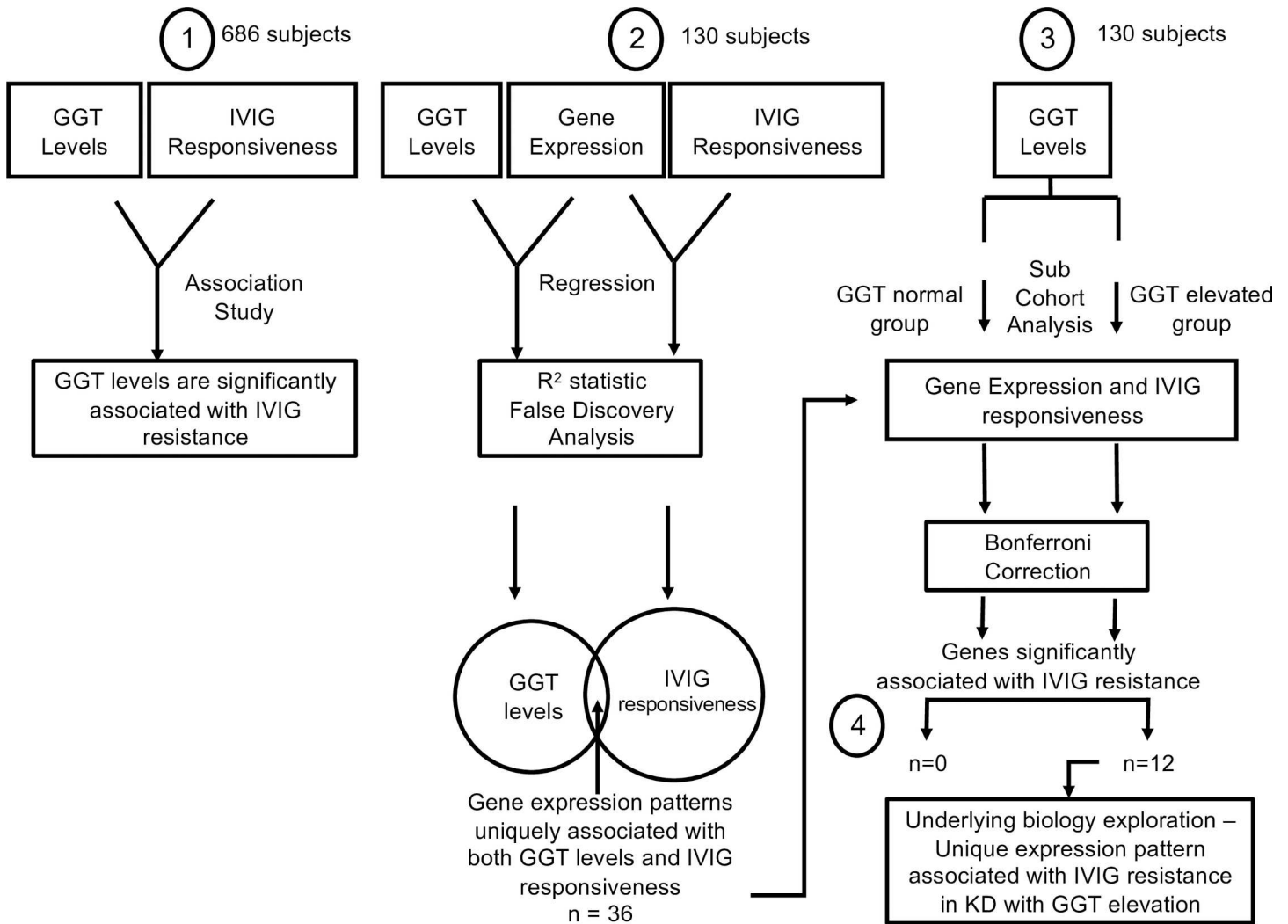


Fig 1. Study outline of analyses to uncover unique gene expression patterns underlying IVIG responsiveness in subjects with elevated serum GGT levels. (1) Analysis of GGT levels with IVIG responsive-ness; (2) Global gene expression analysis by R2 statistic and FDR analyses to identify genes, explaining variations in both IVIG responsiveness and GGT elevation; (3) Targeted analysis of the step 2 discovered genes in either GGT normal or elevated subgroups to reveal gene expression patterns specific for each sub-group of subjects; (4) Pathway and literature analysis to explore the underlying biology of IVIG resistance in subjects with GGT elevation.

doi:10.1371/journal.pone.0167434.g001

obtained from the parents or legal guardians and assent, when appropriate, was obtained from the patient.

To validate our observations on changes of neutrophils in response to IVIG treatment in Cohort III, we utilized another independent cohort (Cohort IV) with 587 KD subjects. Approved by the institutional review board of Korean University Medical Center, the medical records of KD patients from January 2008 to December 2013 were reviewed. The diagnosis and treatment of KD was based on AHA criteria [4]. CAA were diagnosed on the basis of the criteria proposed by the Japanese Kawasaki Disease Research Committee in 1984 [24]. Patients with complete differential leukocyte counts at diagnosis and 2 days after IVIG treatment, IVIG response and CAA data were included. Detailed characteristics, clinical data and results of laboratory studies of subjects were previously described [25].

The absolute neutrophil count (ANC) (mature and immature (band) forms) were calculated for both the UCSD and Korean cohorts.

General data analysis

All statistical analyses and plots were performed using R 3.2.2 [26] and ggplot2 [27] if not mentioned explicitly. The difference in neutrophil reduction between the IVIG responders and the IVIG non-responders after treatment was tested with the Mann-Whitney U-test. The scatter plot curve of neutrophil reduction versus ANC was done with loess fit.

Results

Association between IVIG resistance risk and GGT levels

The 575 IVIG responders and 111 IVIG non-responders in Cohort I had similar demographic and clinical characteristics (Table 1). Subjects were treated with IVIG just after obtaining baseline laboratory values. In addition to elevated GGT levels, IVIG resistant subjects also had significantly higher ALT, CRP values, band percentage, and absolute white blood cell count. This

Table 1. Clinical and laboratory characteristics values of 686 IVIG responsive and resistant KD subjects.

| | IVIG responders (N = 575) | IVIG non-responders (N = 111) | P value |
|---|---------------------------|-------------------------------|---------|
| Age at diagnosis, years | 2.6 (1.4–4.3) | 2.4 (1.4–4.2) | NS |
| Male, N (%) | 350 (60.9) | 72 (64.9) | NS |
| Illness day at sample collection days | 6 (5–7) | 5 (4–6) | NS |
| Incomplete KD, N (%) | 67 (11.7) | 9 (8.1) | NS |
| Coronary artery aneurysms, N (%) | 12 (2.1) | 5 (4.5) | NS |
| Ethnicity, N (%) | | | |
| Asian | 99 (17.2) | 14 (12.6) | NS |
| African-American | 23 (4.0) | 4 (3.6) | NS |
| Caucasian | 135 (23.5) | 30 (27.0) | NS |
| Hispanic | 186 (32.3) | 37 (33.3) | NS |
| More than race | 111 (19.3) | 24 (21.6) | NS |
| Other | 8 (1.3) | 1 (0.9) | NS |
| CRP, mg/dL | 7.0 (4.0–14.6) | 8.3 (5.2–18.6) | < 0.01 |
| ESR, mm/h | 61 (42–77) | 53 (36–68) | < 0.05 |
| WBC, ×10 ³ /mm ³ | 13.2 (10.6–17.3) | 13.6 (10.6–17.4) | NS |
| ANC, cells/mm ³ | 8,836 (6,440–11,696) | 9,585 (7,301–12,328) | NS |
| ZHgb | -1.2 (-2.1–0.42) | -1.2 (-2.2–0) | NS |
| ALT, IU/L | 35 (19–98) | 76 (38–142) | < 0.001 |
| GGT, IU/L | 37 (17–109) | 76 (28–157) | < 0.001 |
| Albumin, g/dL | 3.9 (3.5–4.2) | 3.7 (3.4–4.0) | NS |
| Polymorphonuclear leukocytes (%) | 54 (42–64) | 51.0 (40.0–62.5) | NS |
| Platelet count, ×10 ³ /mm ³ | 373 (291–467) | 340 (282–430) | NS |
| Bands (%) | 11.0 (4.0–19.0) | 18.0 (10.0–32.2) | < 0.001 |
| ABC | 1,440 (554–2,634) | 2,450 (1,272–4,512) | < 0.001 |

Values are presented as median (IQR: Interquartile range). KD: Kawasaki disease, IVIG: intravenous immunoglobulin, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, WBC: white blood count, ANC: absolute neutrophil count, ZHgb: standard deviations from the mean hemoglobin concentration normalized for age, ALT: alanine aminotransferase, GGT: gamma-glutamyl transferase. The difference between groups were tested using Wilcoxon rank sum test, NS: not significant.

doi:10.1371/journal.pone.0167434.t001

is in line with previous meta-analysis [15] that serum levels of ALT and GGT in IVIG non-responders were significantly higher than that in the IVIG responders.

IVIG resistance was examined according to GGT levels quintiles (Fig 2). IVIG resistance risk in each quintile exhibited a progressive association with the GGT levels and the odds ratio reached 2.6 for subjects in the highest quintile compared with the lowest quintile.

Global gene expression analyses with FDR

Of the 130 KD subjects with gene expression data in Cohort II, 100 were IVIG responders and 30 were IVIG non-responders. At the time of diagnosis, 45 subjects had normal serum GGT levels, and 85 had elevated GGT levels (Table C in S1 File). In multiple hypothesis testing of

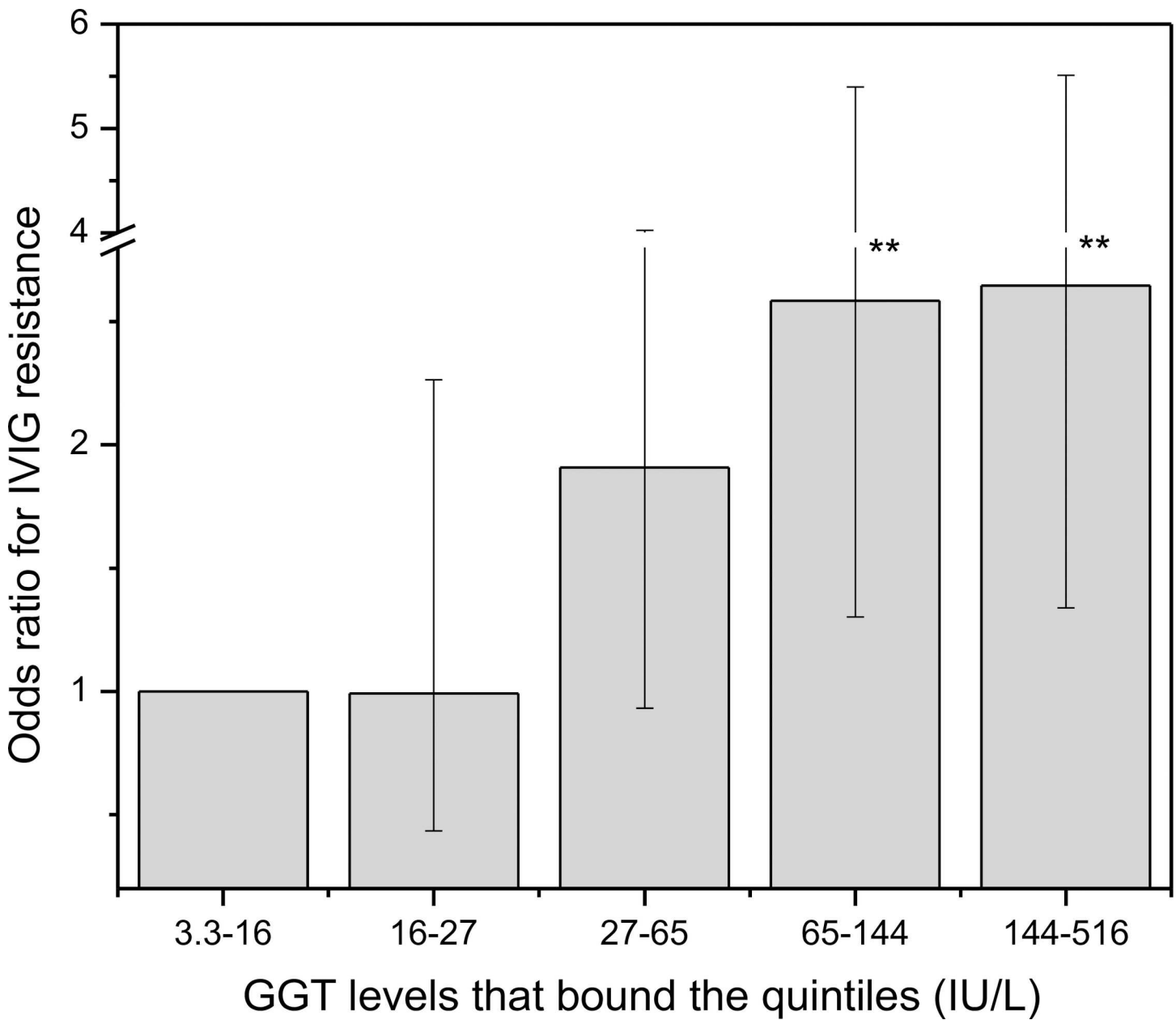


Fig 2. Odds ratio for IVIG resistance per quintile of GGT level. The odds ratios were calculated against the first quintile with the lowest GGT level. The 95% confidential intervals were shown as the error bars. **: *P* value < 0.01 using fisher exact test.

doi:10.1371/journal.pone.0167434.g002

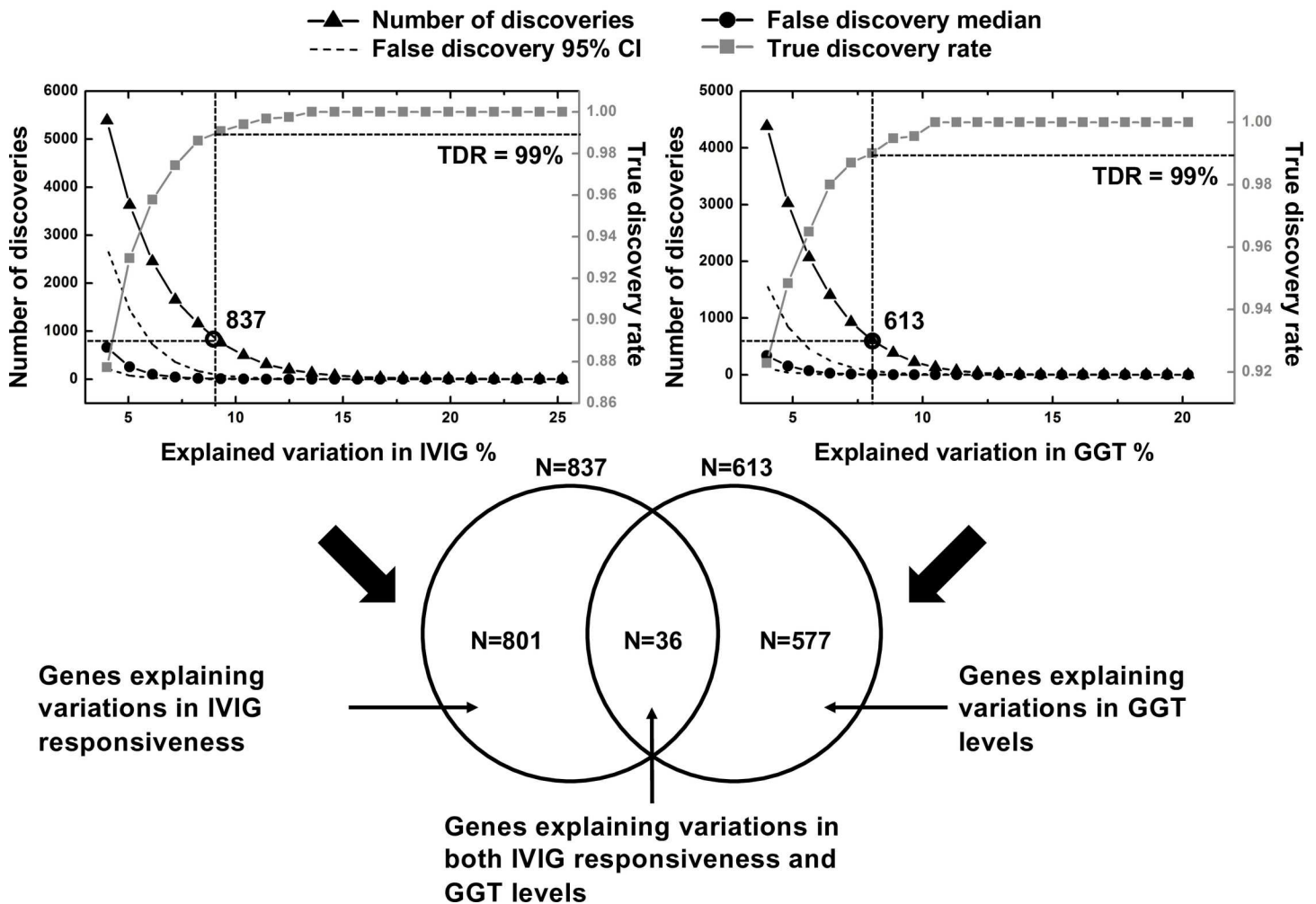


Fig 3. R^2 statistic and FDR analyses with respect to GGT levels and IVIG responsiveness. Top left panel: discovery of genes explaining variations in IVIG responsiveness. Top right panel: discovery of genes explaining variations in GGT levels. Bottom panel: Venn diagram analysis uncovering genes explaining variations in both IVIG responsiveness and GGT levels.

doi:10.1371/journal.pone.0167434.g003

gene expression for GGT levels and IVIG resistance, the number of true discovered genes decreased with increasing R square values (Fig 3). The median of false discoveries decreased much faster than the total number of discoveries because the event, that a gene can explain the significant variations of the randomized response, can only occur by chance. In analyses of IVIG resistance and elevated GGT levels, the R square thresholds corresponding to a TDR of 99% resulted in discovery of 837 and 613 genes, respectively. Of these, 36 were common to both gene sets (Fig 3 bottom panel).

Candidate gene analysis with normal and elevated GGT level sub-cohorts

In order to explore these 36 genes with regard to the GGT-level-associated IVIG responses, we partitioned Cohort II into sub-cohorts with normal (45 subjects including 38 IVIG responders and 7 non-responders) or elevated GGT levels (85 subjects including 62 IVIG responders and 23 non-responders). After applying the Bonferroni correction, significant associations (P value < 0.05) with IVIG resistance persisted for 12 out of the 36 genes among patients with elevated GGT levels and none among patients with normal GGT levels (Table 2). The highest

odds ratio was 114.1 for *ST6GALNAC3* in the GGT-elevated subgroup, and exhibited about a 50-fold difference between the subgroups (Table 2).

Association of the reduced ANC with IVIG treatment outcomes

As shown in Fig 4A, 10 of 95 subjects in Cohort III are IVIG non-responders. Reduction of neutrophil counts after IVIG treatment were common among both IVIG responders (92%) and IVIG non-responders (60%). IVIG non-responders had significantly lower (P value 9.6×10^{-3}) percentage reduction in neutrophil count than responders. A similar pattern was observed in Cohort IV, which included 222 IVIG non-responders and 365 IVIG responders (Fig 4B, P value 1.4×10^{-13}). We further delineated changes in ANC by stratifying patients into quintiles according to the ANC at presentation (S1 Fig). In comparison with non-responders, a significantly greater decline in ANC was observed in responders in every quintile (S1 Fig). The ANC increased in a small fraction of patients in both groups after IVIG treatment: 14.4% of non-responders and 3.5% of responders. We plotted the trending curve of either absolute or percentage of neutrophil reduction against the pretreatment ANC (Cohort III subjects, S2A and S2B Fig; Cohort IV subjects, S2C and S2D Fig). This trending curve showed that neutrophil reduction increased as a function of the pretreatment ANC in both the resistant and responsive subgroups. Thus, Cohort IV analysis validated the Cohort III observations that the IVIG non-responders have less reduction in neutrophil count than the IVIG responders. Within subgroup with elevated GGT levels (S3 Fig), the association between high levels of GGT and neutrophil reduction revealed no clear trend.

Discussion

Our study highlights the significant association of the elevated GGT levels with IVIG treatment outcomes in KD patients. Global gene expression analysis revealed 36 genes that could explain

Table 2. List of 12 genes significantly contributing to IVIG resistance in subgroup with elevated GGT levels identified by logistic regression from 36 genes found by R^2 statistic and FDR analyses.

| Gene symbol | HGNC name | Normal GGT | | Elevated GGT | |
|-------------------|---|---------------------|------------|---------------------|------------|
| | | Odds ratio (95% CI) | P value* | Odds ratio (95% CI) | P value* |
| <i>ST6GALNAC3</i> | ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 3 | 2.3 (0.003–2217) | | 114.1 (9.2–2540) | 0.03 |
| <i>LAMTOR5</i> | late endosomal/lysosomal adaptor, MAPK and MTOR activator 5 | 2.1 (0.3–177.7) | | 112.5 (10–2034) | 0.02 |
| <i>CMTM4</i> | CKLF-like MARVEL transmembrane domain containing 4 | 0.7 (0.01–23.1) | | 34.7 (5.5–303.1) | 0.02 |
| <i>LOC653907</i> | similar to complement component (3b/4b) receptor 1 isoform F precursor (obsolete) | 1.2 (0.1–16.5) | | 19.4 (4.1–119.2) | 0.02 |
| <i>TSHZ3</i> | teashirt zinc finger homeobox 3 | 1.1 (0.2–10.4) | > 0.01 | 14.6 (3.7–72.9) | 0.01 |
| <i>GADD45A</i> | growth arrest and DNA-damage-inducible, alpha | 0.3 (0.04–1.8) | | 12.2 (4.0–49.4) | 0.003 |
| <i>DACH1</i> | dachshund family transcription factor 1 | 0.4 (0.04–3.4) | | 8.8 (2.9–35.0) | 0.02 |
| <i>PCOLCE2</i> | procollagen C-endopeptidase enhancer 2 | 1.2 (0.2–6.4) | | 4.4 (1.9–11.3) | 0.04 |
| <i>MMP8</i> | matrix metalloproteinase 8 | 1.2 (0.2–5.2) | | 2.8 (1.7–5.2) | 0.01 |
| <i>ATP8B2</i> | ATPase, aminophospholipid transporter, class I, type 8B, member 2 | 0.1 (0.005–2.5) | | 0.1 (0.02–0.4) | 0.05 |
| <i>ABCF1</i> | ATP-binding cassette, sub-family F (GCN20), member 1 | 0.2 (0.002–12.0) | | 0.02 (0.0007–0.2) | 0.05 |
| <i>SSBP3</i> | single stranded DNA binding protein 3 | 0.03 (0.0003–1.6) | | 0.03 (0.0003–0.1) | 0.01 |

* P values adjusted by Bonferroni correction.

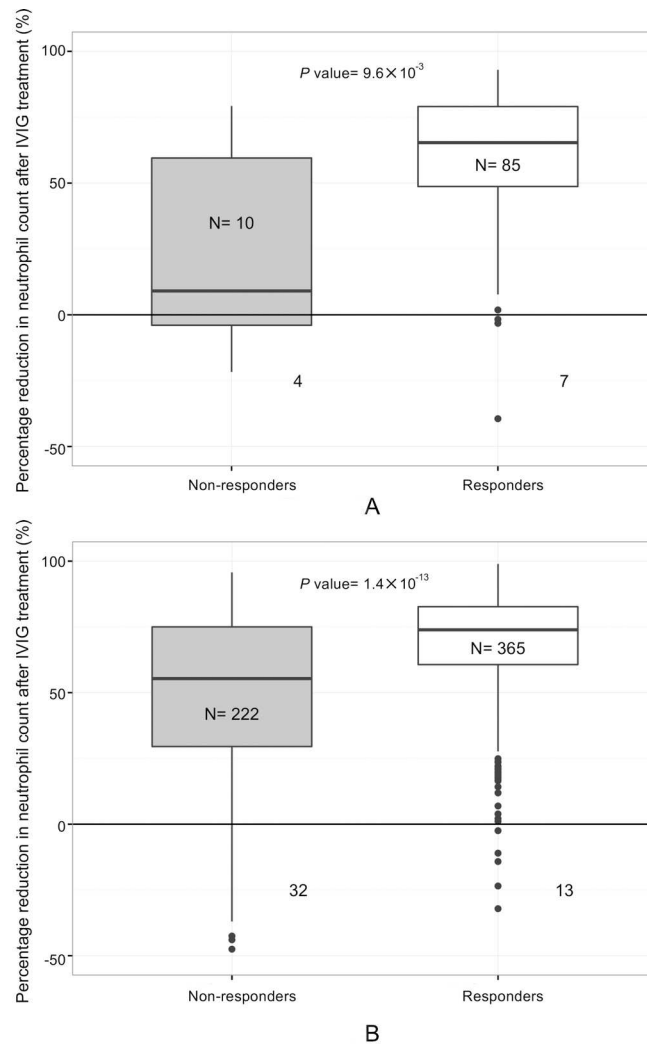


Fig 4. Neutrophil reduction in response to IVIG treatment in two independent cohorts. All *P* values were calculated using Wilcoxon rank sum test. A) Cohort III. B) Cohort IV. The number of patients with rising neutrophil counts after IVIG treatment were indicated by the numbers below x axis alongside each box plot. The *P* values were calculated using Wilcoxon rank sum test.

doi:10.1371/journal.pone.0167434.g004

the variations of both IVIG treatment outcomes and GGT levels in acute KD patients. Of these 36 genes, 12 retained an association with IVIG resistance in the subgroup with elevated GGT levels, while none remained significant in the normal GGT subgroup. In comparison, although both ALT and GGT levels in IVIG resistant patients were significantly higher than that in the IVIG responsive group, none of the 12 GGT-associated genes were significantly associated with IVIG responses in either the normal or elevated ALT subgroups (Table D in [S1 File](#)). These observations suggest that a unique gene expression pattern exists in KD subjects with elevated GGT levels, which may account for their higher risk of resistance to IVIG treatment.

Molecular and immunological markers have been shown to segregate and predict responders and non-responders to IVIG therapy [28]. Consistent with previous observations of significantly elevated neutrophil counts in IVIG-nonresponsive patients, the circulating levels of inflammatory mediators including granulocyte-colony stimulating factor (G-CSF) were significantly higher in IVIG non-responders [29] and were positively correlated with higher levels of matrix metalloproteinase-8 (MMP-8), which is 1 of the 12 GGT associated genes found in this

study [29, 30]. We reasoned that the 12 GGT associated differentially expressed genes were novel, and may provide molecular insight on the IVIG response. We focused the analyses on *ST6GALNAC3*, which was found to have the highest odds ratio in the elevated GGT group and about a 50-fold greater risk compared to the normal GGT group. *ST6GALNAC3* is a sialyltransferase that exclusively utilizes $\alpha 2, 3$ -sialylated ganglioside GM1b as a donor to synthesize ganglioside GD1 α by adding a $\alpha 2, 6$ -sialic acid onto β -galactoside [31]. The branching $\alpha 2, 6$ -sialic acid, could potentially increase the binding affinity of GD1 α to siglec-9, a sialic acid-binding immunoglobulin-type lectin which preferably binds to $\alpha 2,3$ - and $\alpha 2,6$ -sialyl residues [32] on monocytes and neutrophils [33] (Fig 5). We observed that the ANC declined in response to IVIG in both IVIG responders and non-responders (Fig 4B). Given that IVIG non-responders are still febrile with ongoing inflammation, we hypothesized a direct impact of IVIG on neutrophils instead of a secondary therapeutic effect of global inflammation reduction. This is consistent with previous smaller cohort observations [34, 35]. Neutrophils isolated from IVIG responders exhibit accelerated spontaneous apoptosis *in vitro* [36], suggesting that apoptosis may cause the reduction of neutrophils during IVIG treatment. Conversely, neutrophils isolated from IVIG non-responders were less inclined to undergo apoptosis *in vitro* [36]. These observations are in line with our hypothesis that neutrophils in non-responders may be more resistant to IVIG induced apoptosis. In addition, neutrophil count was used as one of the clinical features for IVIG resistance score [37]. Circulating neutrophils and their over activation maybe one of the underlying progressive indicators in IVIG resistance and the severity of the heart lesion [38, 39].

Despite the fact there have been reports of KD occurring in the presence of Autoimmune neutropenia (AIN)[40, 41], for the majority of KD patients, the neutrophil count is elevated. We showed that IVIG non-responders had significantly lower percentage reduction in neutrophil count than responders. The observation of KD symptoms[41] with neutropenia suggested that there might be two KD subgroups with different underlying mechanisms of pathophysiology involving neutrophils. We hypothesize that, within a small subgroup of KD, neutrophil may not necessarily be involved in the pathogenesis and manifestation of KD but rather in the progression and severity of coronary artery lesions (CAL). One explanation in the AIN case with KD is that the neutrophil count sampled in peripheral blood does not always reflect the neutrophil count in the marginated pool or in the tissues. Autopsy data clearly show that neutrophils are the “first responders” in the tissues. Before they can enter the arterial wall, they must marginate. Neither the marginated pool nor the tissue infiltrating neutrophils are measured when blood is drawn. Therefore, it is possible, in the minor subgroup of AIN with KD, neutrophils may still be central to the pathophysiology of the disease even though the numbers measured in the flowing portion of the peripheral blood is low.

Naturally occurring antibodies (Nabs) against siglec-9 (Nabs-siglec-9) are found in IVIG and can induce apoptosis in neutrophils [42–44]. Moreover, this apoptosis is accelerated by proinflammatory cytokines. Granulocyte/macrophage colony-stimulating factor (GM-CSF) and interferon- γ (IFN- γ), which are often upregulated in KD [45, 46]. Therefore, we postulate that Nabs-siglec-9 in IVIG can induce neutrophil apoptosis in KD, explaining the association between the neutrophil reduction and therapeutic outcomes of IVIG (Fig 5): (1) the high expression level of *ST6GALNAC3* leads to increased enzymatic activity of *ST6GALNAC3*, producing more GD1 α , which in turn binds to siglec-9 and prevents its recognition by Nabs-siglec-9 in IVIG; (2) elevation of GGT levels will increase the degradation of extracellular glutathione (GSH) to provide cysteine for *de novo* synthesis of intracellular GSH, reducing the intracellular reactive oxygen species (ROS) in neutrophils. Reduction of ROS in neutrophils decreases neutrophil apoptosis induced by IVIG in the presence of GM-CSF and IFN- γ [44]. However, IVIG works through a myriad of paths. Neutrophil apoptosis is probably one of MANY mechanisms by which IVIG may reduce inflammation.

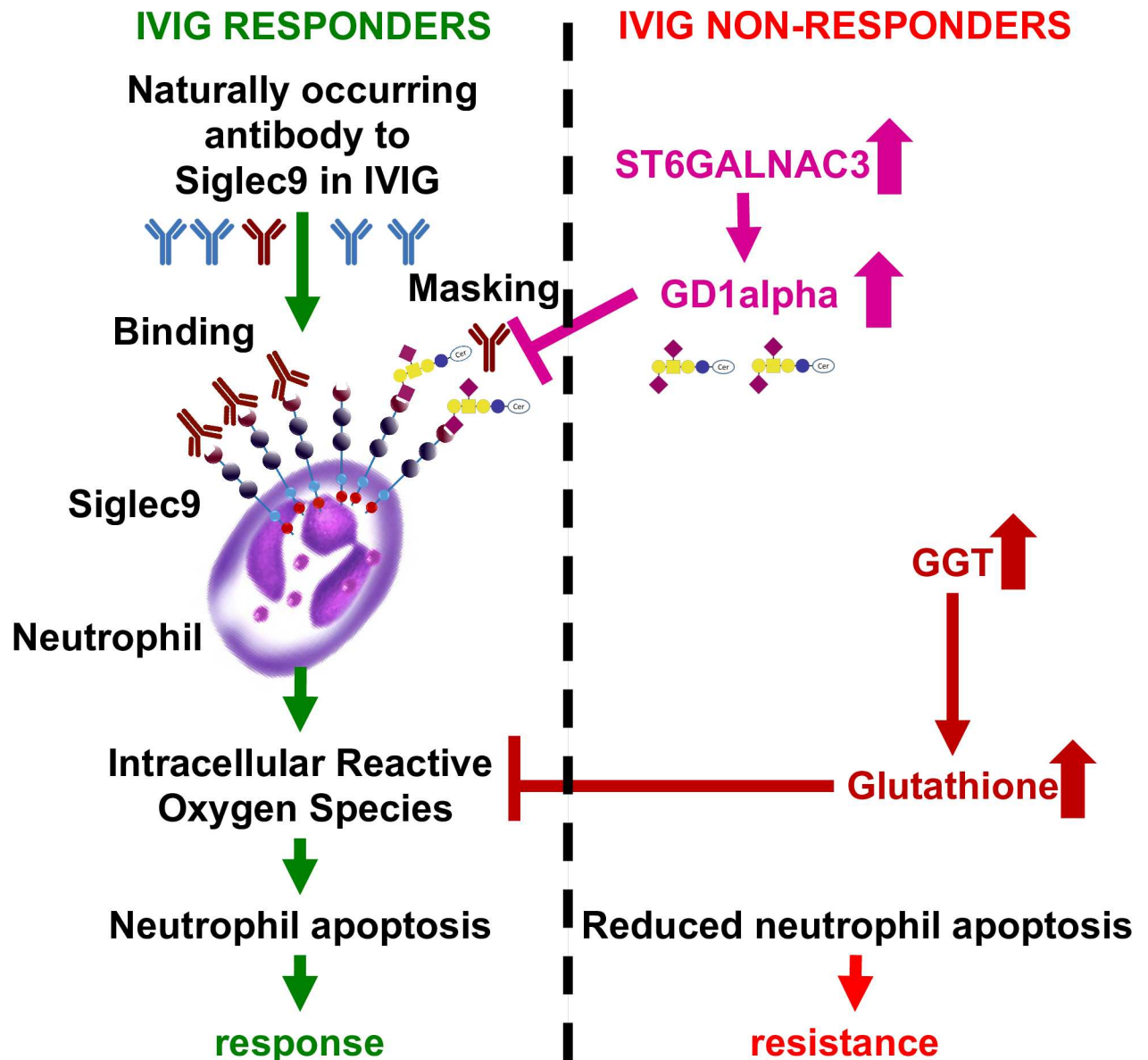


Fig 5. Hypothesis of the underlying biology of IVIG responsiveness involving neutrophils, siglec-9, ST6GALNAC3, and GGT.

doi:10.1371/journal.pone.0167434.g005

Conclusions

To the best of our knowledge, we are the first to integrate multifaceted data sets of expression profiles, clinical parameters and outcomes to explore KD pathophysiology. We demonstrated that KD subjects with elevated GGT levels have a unique gene expression pattern that overlaps with the gene expression pattern associated with IVIG resistance. Our study suggests that reduction of circulating neutrophils is one of the hallmarks of the therapeutic effects of IVIG.

Neutrophil activation and intracellular ROS effect due to elevated GGT levels may not be directly associated with KD susceptibility but be mechanistically critical in IVIG resistance and heart lesion pathogenesis [38, 39, 47]. Therefore, both increased *ST6GALNAC3* and elevated GGT levels may lead to reduced neutrophil apoptosis, and consequently IVIG resistance (Fig 5).

We propose two future testable hypotheses: (1) Nabs against siglec-9 (Nabs-siglec-9) in IVIG can induce apoptosis in neutrophils and contribute to the efficacy of IVIG in the treatment of KD. (2) Increased sialylation of gangliosides block IVIG-mediated neutrophil apoptosis and lead to persistent inflammation and IVIG resistance.

If confirmed, our findings may account for the variable effectiveness of different IVIG lot preparations [48, 49], potentially allowing a new quality control approach. Monoclonal antibodies against Siglec-3 and Siglec-2 are in clinical trials [50]. If developed, therapies to induce neutrophil apoptosis could be a more effective KD treatment.

Supporting Information

S1 Fig. Neutrophil reduction in response to IVIG treatment in different quintiles of ANC before treatment in Cohort IV. Numbers below the x axis are numbers of subjects whose ANC increased after IVIG treatment. Of the 13 IVIG responders with ANC increased after IVIG, 12 fell into the lowest quintile of acute phase neutrophils, and none in the last three quintiles.

(TIF)

S2 Fig. Loess curve analyses of neutrophil reduction or the percentage reduction in neutrophil count as a function of the pretreatment ANC (A-B: Cohort III; C-D: Cohort IV).

(TIFF)

S3 Fig. The percentage reduction in neutrophil count as a function of the IVIG GGT levels in subjects with elevated GGT serum levels.

(TIF)

S1 File. Table A in S1 File. The standard range for normal GGT (IU/L); Table B in S1 File. The standard range for normal ALT (IU/L); Table C in S1 File. The clinical characteristics and laboratory values for subjects selected for gene expression analysis; Table D in S1 File. Association of 12 genes with IVIG response in subgroup with normal and elevated CRP levels; Table E in S1 File. Association of 12 genes with IVIG response in subgroup with normal and elevated CRP levels.

(PPTX)

Acknowledgments

We are very grateful for the contribution of the Pediatric Emergency Medicine Kawasaki Disease Research Group.

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References

1. Uehara R, Belay ED. Epidemiology of Kawasaki disease in Asia, Europe, and the United States. *J Epidemiol.* 2012; 22(2):79–85. PubMed Central PMCID: PMC3798585. doi: [10.2188/jea.JE20110131](https://doi.org/10.2188/jea.JE20110131) PMID: [22307434](https://pubmed.ncbi.nlm.nih.gov/22307434/)
2. Kato H, Sugimura T, Akagi T, Sato N, Hashino K, Maeno Y, et al. Long-term consequences of Kawasaki disease. A 10- to 21-year follow-up study of 594 patients. *Circulation.* 1996; 94(6):1379–85. Epub 1996/09/15. PMID: [8822996](https://pubmed.ncbi.nlm.nih.gov/8822996/)
3. Burns JC, Glode MP. Kawasaki syndrome. *Lancet.* 2004; 364(9433):533–44. Epub 2004/08/11. doi: [10.1016/S0140-6736\(04\)16814-1](https://doi.org/10.1016/S0140-6736(04)16814-1) PMID: [15302199](https://pubmed.ncbi.nlm.nih.gov/15302199/)
4. Newburger JW, Takahashi M, Gerber MA, Gewitz MH, Tani LY, Burns JC, et al. Diagnosis, treatment, and long-term management of Kawasaki disease: a statement for health professionals from the Committee on Rheumatic Fever, Endocarditis and Kawasaki Disease, Council on Cardiovascular Disease in the Young, American Heart Association. *Circulation.* 2004; 110(17):2747–71. doi: [10.1161/01.CIR.0000145143.19711.78](https://doi.org/10.1161/01.CIR.0000145143.19711.78) PMID: [15505111](https://pubmed.ncbi.nlm.nih.gov/15505111/)
5. Newburger JW, Takahashi M, Beiser AS, Burns JC, Bastian J, Chung KJ, et al. A single intravenous infusion of gamma globulin as compared with four infusions in the treatment of acute Kawasaki syndrome. *The New England journal of medicine.* 1991; 324(23):1633–9. Epub 1991/06/06. doi: [10.1056/NEJM199106063242305](https://doi.org/10.1056/NEJM199106063242305) PMID: [1709446](https://pubmed.ncbi.nlm.nih.gov/1709446/)
6. Ogata S, Tremoulet AH, Sato Y, Ueda K, Shimizu C, Sun X, et al. Coronary artery outcomes among children with Kawasaki disease in the United States and Japan. *Int J Cardiol.* 2013; 168(4):3825–8. PubMed Central PMCID: PMC4002741. doi: [10.1016/j.ijcard.2013.06.027](https://doi.org/10.1016/j.ijcard.2013.06.027) PMID: [23849968](https://pubmed.ncbi.nlm.nih.gov/23849968/)
7. Newburger JW, Takahashi M, Burns JC, Beiser AS, Chung KJ, Duffy CE, et al. The treatment of Kawasaki syndrome with intravenous gamma globulin. *The New England journal of medicine.* 1986; 315(6):341–7. Epub 1986/08/07. doi: [10.1056/NEJM198608073150601](https://doi.org/10.1056/NEJM198608073150601) PMID: [2426590](https://pubmed.ncbi.nlm.nih.gov/2426590/)
8. Durongpisitkul K, Gururaj VJ, Park JM, Martin CF. The prevention of coronary artery aneurysm in Kawasaki disease: a meta-analysis on the efficacy of aspirin and immunoglobulin treatment. *Pediatrics.* 1995; 96(6):1057–61. Epub 1995/12/01. PMID: [7491221](https://pubmed.ncbi.nlm.nih.gov/7491221/)
9. Burns JC, Capparelli EV, Brown JA, Newburger JW, Glode MP. Intravenous gamma-globulin treatment and retreatment in Kawasaki disease. US/Canadian Kawasaki Syndrome Study Group. *The Pediatric infectious disease journal.* 1999; 17(12):1144–8. Epub 01/07.
10. Tremoulet AH, Best BM, Song S, Wang S, Corinaldesi E, Eichenfield JR, et al. Resistance to intravenous immunoglobulin in children with Kawasaki disease. *The Journal of pediatrics.* 2008; 153(1):117–21. Epub 2008/06/24. PubMed Central PMCID: PMC2526555. doi: [10.1016/j.jpeds.2007.12.021](https://doi.org/10.1016/j.jpeds.2007.12.021) PMID: [18571548](https://pubmed.ncbi.nlm.nih.gov/18571548/)
11. Tremoulet AH, Jain S, Chandrasekar D, Sun X, Sato Y, Burns JC. Evolution of Laboratory Values in Patients with Kawasaki Disease. *The Pediatric infectious disease journal.* 2011; 30(12):1022–6. doi: [10.1097/INF.0b013e31822d4f56](https://doi.org/10.1097/INF.0b013e31822d4f56) PMID: [21817952](https://pubmed.ncbi.nlm.nih.gov/21817952/)

12. Ting EC, Capparelli EV, Billman GF, Lavine JE, Matsubara T, Burns JC. Elevated gamma-glutamyl-transferase concentrations in patients with acute Kawasaki disease. *The Pediatric infectious disease journal*. 1998; 17(5):431–2. Epub 06/05. PMID: [9613663](#)
13. Fukunishi M, Kikkawa M, Hamana K, Onodera T, Matsuzaki K, Matsumoto Y, et al. Prediction of non-responsiveness to intravenous high-dose gamma-globulin therapy in patients with Kawasaki disease at onset. *The Journal of pediatrics*. 2000; 137(2):172–6. doi: [10.1067/mpd.2000.104815](#) PMID: [10931407](#)
14. Fu PP, Du ZD, Pan YS. Novel predictors of intravenous immunoglobulin resistance in Chinese children with Kawasaki disease. *The Pediatric infectious disease journal*. 2013; 32(8):e319–23. Epub 03/01. doi: [10.1097/INF.0b013e31828e887f](#) PMID: [23446442](#)
15. Liu L, Yin W, Wang R, Sun D, He X, Ding Y. The prognostic role of abnormal liver function in IVIG unresponsiveness in Kawasaki disease: a meta-analysis. *Inflammation research: official journal of the European Histamine Research Society [et al]*. 2015. Epub 12/10.
16. Hoang LT, Shimizu C, Ling L, Naim AN, Khor CC, Tremoulet AH, et al. Global gene expression profiling identifies new therapeutic targets in acute Kawasaki disease. *Genome medicine*. 2014; 6(11):541. Epub 2015/01/24. PubMed Central PMCID: PMC4279699. doi: [10.1186/s13073-014-0102-6](#) PMID: [25614765](#)
17. Marschner IC, others. glm2: fitting generalized linear models with convergence problems. *The R journal*. 2011; 3:12–5.
18. McFadden D. Conditional logit analysis of qualitative choice behavior. In: Zarembka P, editor. *Frontiers in Econometrics*: Academic Press; 1973. p. 105–42.
19. Long JS. *Regression Models for Categorical and Limited Dependent Variables*: Sage; 1997. 104–6 p.
20. Ling XB, Cohen H, Jin J, Lau I, Schilling J. FDR made easy in differential feature discovery and correlation analyses. *Bioinformatics*. 2009; 25(11):1461–2. doi: [10.1093/bioinformatics/btp176](#) PMID: [19376824](#)
21. Bonferroni CE. *Teoria statistica delle classi e calcolo delle probabilità*. Pubblicazioni del R Istituto Superiore di Scienze Economiche e Commerciali di Firenze. 1936; 8:3–62.
22. Miller RGJ. *Simultaneous Statistical Inference*. New York: Springer-Verlag; 1991.
23. Tremoulet AH, Jain S, Jaggi P, Jimenez-Fernandez S, Pancheri JM, Sun X, et al. Infliximab for intensification of primary therapy for Kawasaki disease: a phase 3 randomised, double-blind, placebo-controlled trial. *Lancet*. 2014; 383(9930):1731–8. Epub 2014/02/28. doi: [10.1016/S0140-6736\(13\)62298-9](#) PMID: [24572997](#)
24. Report of Subcommittee on Standardization of Diagnostic Criteria and Reporting of Coronary Artery Lesions in Kawasaki Disease. In: Ministry of Health and Welfare, editor.: *Research Committee of Kawasaki Disease*; 1984.
25. Ha KS, Lee J, Jang GY, Lee J, Lee KC, Son CS, et al. Value of neutrophil-lymphocyte ratio in predicting outcomes in Kawasaki disease. *Am J Cardiol*. 2015; 116(2):301–6. doi: [10.1016/j.amjcard.2015.04.021](#) PMID: [25975725](#)
26. R Core Team. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing; 2015.
27. Wickham H. *ggplot2: elegant graphics for data analysis*: Springer New York; 2009.
28. Galeotti C, Kaveri SV, Bayry J. Molecular and immunological biomarkers to predict IVIg response. *Trends Mol Med*. 2015; 21(3):145–7. doi: [10.1016/j.molmed.2015.01.005](#) PMID: [25680699](#)
29. Abe J, Ebata R, Jibiki T, Yasukawa K, Saito H, Terai M. Elevated granulocyte colony-stimulating factor levels predict treatment failure in patients with Kawasaki disease. *The Journal of allergy and clinical immunology*. 2008; 122(5):1008–13 e8. Epub 10/22. doi: [10.1016/j.jaci.2008.09.011](#) PMID: [18930517](#)
30. Fury W, Tremoulet AH, Watson VE, Best BM, Shimizu C, Hamilton J, et al. Transcript abundance patterns in Kawasaki disease patients with intravenous immunoglobulin resistance. *Human immunology*. 2010; 71(9):865–73. Epub 07/06. PubMed Central PMCID: PMC42929310. doi: [10.1016/j.humimm.2010.06.008](#) PMID: [20600450](#)
31. Tsuchida A, Ogiso M, Nakamura Y, Kiso M, Furukawa K, Furukawa K. Molecular cloning and expression of human ST6GalNAc III: restricted tissue distribution and substrate specificity. *J Biochem*. 2005; 138(3):237–43. doi: [10.1093/jb/mvi124](#) PMID: [16169874](#)
32. Yamaji T, Teranishi T, Alphey MS, Crocker PR, Hashimoto Y. A small region of the natural killer cell receptor, Siglec-7, is responsible for its preferred binding to alpha 2,8-disialyl and branched alpha 2,6-sialyl residues. A comparison with Siglec-9. *J Biol Chem*. 2002; 277(8):6324–32. doi: [10.1074/jbc.M110146200](#) PMID: [11741958](#)
33. Zhang JQ, Nicoll G, Jones C, Crocker PR. Siglec-9, a novel sialic acid binding member of the immunoglobulin superfamily expressed broadly on human blood leukocytes. *J Biol Chem*. 2000; 275(29):22121–6. doi: [10.1074/jbc.M002788200](#) PMID: [10801862](#)

34. Hwang JY, Lee KY, Rhim JW, Youn YS, Oh JH, Han JW, et al. Assessment of intravenous immunoglobulin non-responders in Kawasaki disease. *Arch Dis Child*. 2011; 96(11):1088–90. doi: [10.1136/adc.2010.184101](https://doi.org/10.1136/adc.2010.184101) PMID: [20551193](https://pubmed.ncbi.nlm.nih.gov/20551193/)
35. Lee SM, Lee JB, Go YB, Song HY, Lee BJ, Kwak JH. Prediction of resistance to standard intravenous immunoglobulin therapy in kawasaki disease. *Korean Circ J*. 2014; 44(6):415–22. PubMed Central PMCID: [PMCPMC4248614](https://pubmed.ncbi.nlm.nih.gov/25469144/). doi: [10.4070/kcj.2014.44.6.415](https://doi.org/10.4070/kcj.2014.44.6.415) PMID: [25469144](https://pubmed.ncbi.nlm.nih.gov/25469144/)
36. Tsujimoto H, Takeshita S, Nakatani K, Kawamura Y, Tokutomi T, Sekine I. Intravenous immunoglobulin therapy induces neutrophil apoptosis in Kawasaki disease. *Clin Immunol*. 2002; 103(2):161–8. doi: [10.1006/clim.2002.5209](https://doi.org/10.1006/clim.2002.5209) PMID: [12027421](https://pubmed.ncbi.nlm.nih.gov/12027421/)
37. Kobayashi T, Inoue Y, Takeuchi K, Okada Y, Tamura K, Tomomasa T, et al. Prediction of intravenous immunoglobulin unresponsiveness in patients with Kawasaki disease. *Circulation*. 2006; 113(22):2606–12. Epub 2006/06/01. doi: [10.1161/CIRCULATIONAHA.105.592865](https://doi.org/10.1161/CIRCULATIONAHA.105.592865) PMID: [16735679](https://pubmed.ncbi.nlm.nih.gov/16735679/)
38. Kanai T, Ishiwata T, Kobayashi T, Sato H, Takizawa M, Kawamura Y, et al. Ulinastatin, a urinary trypsin inhibitor, for the initial treatment of patients with Kawasaki disease: a retrospective study. *Circulation*. 2011; 124(25):2822–8. doi: [10.1161/CIRCULATIONAHA.111.028423](https://doi.org/10.1161/CIRCULATIONAHA.111.028423) PMID: [22104548](https://pubmed.ncbi.nlm.nih.gov/22104548/)
39. Biezeveld MH, van Mierlo G, Lutter R, Kuipers IM, Dekker T, Hack CE, et al. Sustained activation of neutrophils in the course of Kawasaki disease: an association with matrix metalloproteinases. *Clin Exp Immunol*. 2005; 141(1):183–8. PubMed Central PMCID: [PMCPMC1809423](https://pubmed.ncbi.nlm.nih.gov/1809423/). doi: [10.1111/j.1365-2249.2005.02829.x](https://doi.org/10.1111/j.1365-2249.2005.02829.x) PMID: [15958085](https://pubmed.ncbi.nlm.nih.gov/15958085/)
40. Ueno K, Nomura Y, Arata M, Maruyama S, Tanabe T, Eguchi T, et al. Development of Kawasaki syndrome in autoimmune neutropenia after treatment with granulocyte colony-stimulating factor. *Pediatr Int*. 2011; 53(3):388–90. doi: [10.1111/j.1442-200X.2011.03376.x](https://doi.org/10.1111/j.1442-200X.2011.03376.x) PMID: [21696506](https://pubmed.ncbi.nlm.nih.gov/21696506/)
41. Okada S, Hasegawa S, Suzuki Y, Ichimura T, Kaneyasu H, Shimomura M, et al. Remission of autoimmune neutropenia after development of Kawasaki disease. *Pediatr Int*. 2015; 57(5):1012–4. doi: [10.1111/ped.12701](https://doi.org/10.1111/ped.12701) PMID: [26508185](https://pubmed.ncbi.nlm.nih.gov/26508185/)
42. von Gunten S, Simon HU. Natural anti-Siglec autoantibodies mediate potential immunoregulatory mechanisms: implications for the clinical use of intravenous immunoglobulins (IVIg). *Autoimmun Rev*. 2008; 7(6):453–6. doi: [10.1016/j.autrev.2008.03.015](https://doi.org/10.1016/j.autrev.2008.03.015) PMID: [18558361](https://pubmed.ncbi.nlm.nih.gov/18558361/)
43. Schaub A, von Gunten S, Vogel M, Wymann S, Rueggsegger M, Stadler BM, et al. Dimeric IVIG contains natural anti-Siglec-9 autoantibodies and their anti-idiotypes. *Allergy*. 2011; 66(8):1030–7. doi: [10.1111/j.1398-9995.2011.02579.x](https://doi.org/10.1111/j.1398-9995.2011.02579.x) PMID: [21385183](https://pubmed.ncbi.nlm.nih.gov/21385183/)
44. von Gunten S, Schaub A, Vogel M, Stadler BM, Miescher S, Simon HU. Immunologic and functional evidence for anti-Siglec-9 autoantibodies in intravenous immunoglobulin preparations. *Blood*. 2006; 108(13):4255–9. doi: [10.1182/blood-2006-05-021568](https://doi.org/10.1182/blood-2006-05-021568) PMID: [16902148](https://pubmed.ncbi.nlm.nih.gov/16902148/)
45. Matsubara T, Furukawa S, Yabuta K. Serum levels of tumor necrosis factor, interleukin 2 receptor, and interferon-gamma in Kawasaki disease involved coronary-artery lesions. *Clin Immunol Immunopathol*. 1990; 56(1):29–36. PMID: [2113446](https://pubmed.ncbi.nlm.nih.gov/2113446/)
46. Igarashi H, Hatake K, Tomizuka H, Yamada M, Gunji Y, Momoi MY. High serum levels of M-CSF and G-CSF in Kawasaki disease. *Br J Haematol*. 1999; 105(3):613–5. PMID: [10354120](https://pubmed.ncbi.nlm.nih.gov/10354120/)
47. Yoshimura K, Tatsumi K, Iharada A, Tsuji S, Tateiwa A, Teraguchi M, et al. Increased nitric oxide production by neutrophils in early stage of Kawasaki disease. *European journal of pediatrics*. 2009; 168(9):1037–41. doi: [10.1007/s00431-008-0872-1](https://doi.org/10.1007/s00431-008-0872-1) PMID: [19020897](https://pubmed.ncbi.nlm.nih.gov/19020897/)
48. Tsai MH, Huang YC, Yen MH, Li CC, Chiu CH, Lin PY, et al. Clinical responses of patients with Kawasaki disease to different brands of intravenous immunoglobulin. *The Journal of pediatrics*. 2006; 148(1):38–43. doi: [10.1016/j.jpeds.2005.08.024](https://doi.org/10.1016/j.jpeds.2005.08.024) PMID: [16423595](https://pubmed.ncbi.nlm.nih.gov/16423595/)
49. Lin MC, Fu YC, Jan SL, Lai MS. Comparative effectiveness of intravenous immunoglobulin for children with Kawasaki disease: a nationwide cohort study. *PLoS One*. 2013; 8(5):e63399. PubMed Central PMCID: [PMCPMC3641142](https://pubmed.ncbi.nlm.nih.gov/3641142/). doi: [10.1371/journal.pone.0063399](https://doi.org/10.1371/journal.pone.0063399) PMID: [23650564](https://pubmed.ncbi.nlm.nih.gov/23650564/)
50. Jandus C, Simon HU, von Gunten S. Targeting siglecs—a novel pharmacological strategy for immunotherapy. *Biochem Pharmacol*. 2011; 82(4):323–32. doi: [10.1016/j.bcp.2011.05.018](https://doi.org/10.1016/j.bcp.2011.05.018) PMID: [21658374](https://pubmed.ncbi.nlm.nih.gov/21658374/)