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Neutrophil Extracellular Traps Formation and Citrullinated Histones 3 Levels in Patients with Kawasaki Disease

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ABSTRACT

Background: Kawasaki disease (KD) is a vasculitis associated with vascular injury and autoimmune response. Inflammatory factors stimulate neutrophils to produce web-like structures called neutrophil extracellular traps (NETs). Citrullinated histone 3 (H3Cit) is one of the main protein components of neutrophil extracellular traps involved in the process of NETosis. The levels of NETs and H3Cit in the KD are not known.

Objective: To determine the changes in the levels of NETs and H3Cit in KD.

Methods: Children with KD were recruited and divided into the acute KD and the sub-acute KD group according to the disease phase and whether intravenous immunoglobulin (IVIG) was used or not. Peripheral venous blood was taken before and after the IVIG administration and sent for the examination of NETs by flow cytometry. The level of H3Cit was measured by enzyme-linked immunosorbent assay (ELISA).

Results: The counts of NETs in the acute KD group significantly increased compared with the healthy controls (p<0.01). The level of H3Cit was significantly higher in the acute KD group than in the healthy control subjects. Of note, both the counts of NETs and the level of H3Cit decreased in the KD patients treated with IVIG compared with the acute KD group (p<0.01).

Conclusion: Acute KD is characterized by an increased formation of NETs and high levels of H3Cit. IVIG significantly inhibited NETs formation and also reduced the level of plasma H3Cit in children with KD.

Keywords: Citrullinated Histone 3, IVIG, Kawsaki Disease, Neutrophil Extracellular Traps

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INTRODUCTION

Kawasaki disease (KD) is a kind of systemic self-limited vasculitis, associated with vascular injury and autoimmune response (1). It has become the leading cause of acquired heart disease in children (2). In 2020, the number of children with KD and Kawasaki-like disease after Corona Virus Disease 2019 (COVID-19) infection increased significantly in many countries (3, 4). The efficacy of intravenous immunoglobulin (IVIG) administered in the acute phase of KD is well-established, but the immune mechanism of IVIG has not been fully revealed. However, the etiology and the true worldwide incidence, morbidity, and mortality will remain unknown until a diagnostic test is developed (5, 6). In 2004, the American Heart Association suggested that KD is a risk factor for coronary heart disease in adulthood (7). Therefore, the persistent vascular endothelial injury and dysfunction of KD are closely related to the long-term clinical outcome (8). Neutrophils are present in vascular endothelial cells and participate in inflammatory reactions (9, 10). Our previous experiments demonstrated that the activation of neutrophils may play a significant role in the pathogenesis of coronary artery lesions (CALs) in KD (11).

Neutrophils produce web-like structures composed of decondensed chromatin and antimicrobial proteins called neutrophil extracellular traps (NETs), which can neutralize and eliminate pathogens to prevent them from spreading (12). More and more evidence indicated that the NETs serve as effective antimicrobial defenses, but also as putative sources of molecules with immune effector and pro-inflammatory roles. In susceptible individuals, NETs may be the autoantigen of various autoimmune diseases and may promote tissue damage and autoimmunity (13). In various lung diseases, cancer, and cardiovascular diseases, the excessive production and/or reduction of degradation of NETs play a key role in the initiation and persistence of autoimmune response and organ damage (14-16). Since KD is related to vascular injury and autoimmune response, the NETs may be involved in the pathophysiology of KD. Citrullinated histone 3 (H3Cit) is one of the main protein components of neutrophil extracellular traps, highly involved in the process of the NETs (17); therefore, it is considered to be a good biomarker of the NETs. H3Cit can be used as a therapeutic target in septic mice and can reduce the inflammatory response of sepsis (18). H3Cit is secreted in the form of microvesicles and affects the circulatory system by binding with platelets (19). It plays a certain role in thrombotic diseases and microcirculatory disorders (20). Nevertheless, the role of H3Cit in the pathogenesis of KD has not been yet clarified.

The primary objective of this study was to investigate the changes of NETs formation and H3Cit level in KD patients before and after IVIG treatment, and the relationship between NETs and H3Cit.

MATERIALS AND METHODS

Patients and Sample Collection

Children with KD were recruited from January 2021 to December 2021 at the Wuxi Children's Hospital (Wuxi, China). All the patients have excreted infection with COVID-19. The diagnostic basis was based on the standards revised by the fifth KD Committee of Japan. Children with a disease course of more than 10 days or who have been treated with IVIG were ruled out of research subjects. Once the patients met the diagnostic criteria of KD, all of them were treated with oral aspirin (30-50mg/kg per day), and IVIG (2 g/kg per day) immediately. Acute KD means the acute phase from days 1 to 10 in the disease process, characterized by a necrotizing vasculitis with a predominance of neutrophils before the treatment of IVIG. Sub-acute KD starts from 11 days and is characterized by resolution of fever (<37.5 °C) for 48 h after finishing the initial

IVIG treatment. The healthy control group comprised children of the same age visiting our hospital for health examination and did not exhibit any abnormalities in medical examination. After admission, blood samples were taken from all the acute and sub-acute KD patients and the healthy controls.

This study accorded with the ethical standards formulated by the Committee in charge of human trials at the Children's Hospital of WUXI. (Approval No. WXCH2021-02-004). The study was approved by the Committee, and informed consent was obtainded from the parents or authorized legal representatives of all the subjects.

Peripheral Blood Neutrophil Extraction

Two ml of peripheral venous blood was taken from both the acute and sub-acute KD subjects and sent for examination of NETs within 2 h using flow cytometry. Neutrophil Extraction Kit (Solarbio, Beijing Solarbio Science & Technology Co, Beijing, China) was used for peripheral blood polymorphonuclear neutrophils (PMNs) isolation according to the manufacturing structure. The viability (≥95%) of the cells was confirmed by a TC-20-automated cell counter (Bio-Rad Laboratories, Hercules, CA, USA). Neutrophils were cultured with 6% serum obtained from venous blood in 5% CO2 at 37 °C for 3 h.

Assessment of NETs Production

All blood samples of the 7 KD patients (3 male/4 female) and the 7 healthy control subjects were collected both before and after the IVIG injection. The flow cytometry protocol was adapted from the protocol published by Gavillet M. et al. (21). PMNs were seeded at a density of 1×105/100 μl in 24 hole plates, the 24-well plates were taken out, 1ml of 4% paraformaldehyde was added directly and fixed for 20 min (BALF can start directly from this step), centrifugated for 20 min with 12000 rpm, and then the supernatant was discarded. Then, it was washed with 1 ml phosphate buffer solution (PBS), centrifuged for 20 min, and the supernatant was discarded; next it was resuspended to 100ul; anti-Histone 3pS28-APC was added (130-105-701, h&m, 30 tests) at 1:300 dilution, and FITC-conjugated anti-myeloperoxidase (MPO) antibody (ab90812-50ug [2D4]) was analyzed by flow cytometry. Then, it was kept away from light and room temperature for 30 min; 1ml PBS was added for cleaning, centrifuged for 20 min, and the supernatant was discarded; it was incubated sequentially and resuspended in 2% BSA. Then, it was analyzed by flow-cytometry according to the gating strategy detailed by Gavillet M et al. Anti-MPO- and anti-H3-Histone-antibody positive cells were defined as surrogates for NETs.

Evaluation of H3Cit Levels

The level of H3Cit in serum samples of 27 acute KD, 20 sub-acute KD patients, and 20 healthy children was measured. 2 ml of peripheral blood acute KD and sub-acute KD subjects was anticoagulated with EDTA, centrifuged at 4 ℃ ×3000 g for 20 min., the supernatant was taken and frozen at -80 ℃ for the measurement of H3Cit using enzyme-linked immunosorbent assay (ELISA). H3Cit was analyzed by a commercial sandwich ELISA kit (Cayman Chemical, Ann Arbor, Michigan, USA 501620-96wells) and performed according to the manufacturer's instructions. This H3Cit assay employed a monoclonal antibody specific for histone H3 citrullinated at R2, R8, and R17 (clone 11D3).

Statistical Analysis

BD facsdiva version 6.1.3 (BD Biosciences, NJ, US) software was used on BD facscanto II (BD Biosciences, NJ, US), and flowj (BD, US) was used to analyze the flow cytometry data. The analyses were carried out using statistical software package version 12.5 (TIBCO Inc). The classification variables are expressed by frequency and rate, and the difference analysis is conducted by Chi-squared test ($\chi 2$). The continuity variables are first tested for normality. If the data

meet the normal distribution in different groups at the same time (p>0.05), the data distribution is expressed by the means±EM, and the difference analysis is performed by one-way ANOVA. The level of H3Cit was reported as an interquartile range. For the statistical analysis of the differences between the study groups, the Kruskal-Wallis test was performed followed by the Mann-Whitney test for the analysis of intergroup differences. The results were considered significant at *p≤0.05 and **p≤0.01.

RESULTS

IVIG Inhibited NETs Formation in the KD Group

There are no significant differences in gender or age among the three groups. The levels of white blood cell count /neutrophils (%) and c-reactive protein (CRP) in the KD group were higher than that in the healthy control (HC) group. The Platelet count of sub-acute KD was higher than that in the acute KD and HC group, consistent with the characteristics of KD (Table 1).

The acute KD group (Fig. 1) showed significantly higher NETs counts than the sub-acute KD (Fig. 1b, 28.757±6.336 vs 12.794±2.804, p<0.01) and HC groups (Fig. 1c, 12.794±2.804 vs 10.973±3.659, p<0.01). After IVIG treatment, the NETs count decreased in KD patients. There is no significant difference between the sub-acute KD and HC groups (Fig. 2).

IVIG Downregulated the Level of H3Cit

The clinical and laboratory characteristics of the studied subjects are given in Table 2. KD individuals were characterized by increased levels of CRP, white blood cell and

Table 1. Comparison of NETs detection data and clinical characteristics among three groups.

Characteristics	Acute KD	Sub-acute KD	HC	*P*
Male (n)	3	3	4	NS
Age at onset (m)	15.90±7.730	15.90±7.730	11.20±3.808	NS
CRP (mg/dl)	70.07±23.10	10.94±0.9079	3.343±0.6043	**
White blood cell count ($\times10^9$ /l)	15.63±1.328	9.423±0.7834	5.851±0.4816	**
Neutrophils ($\times10^9$ /l)	10.49±1.489	4.763±0.5841	2.386±0.265	**
Platelet count ($\times10^9$ /l)	298.1±22.34	370.0±30.70	260.4±30.34	NS
NETs (%)	28.757±6.336	12.794±2.804	10.973±3.659	**

Neutrophil extracellular traps (NETs); Kawasaki disease (KD); Healthy control (HC); C-reactive protein (CRP)

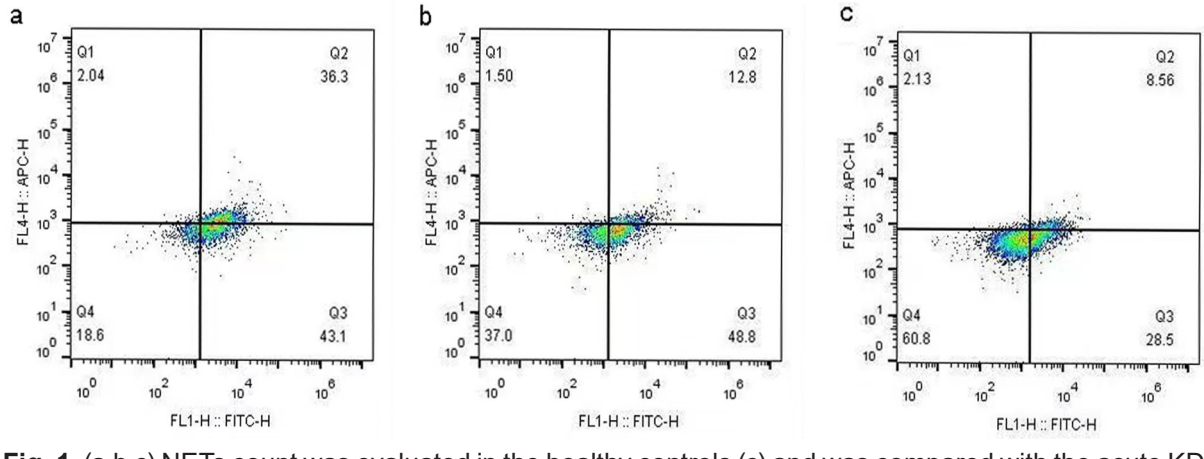

Fig. 1. (a,b,c) NETs count was evaluated in the healthy controls (c) and was compared with the acute KD (a) or sub-KD (b) samples. Flow data showing the initial FSC/SSC blot, anti-MPO (FL1-H:FITC), and anti-H3-Histone (FL4-H:APC-H) double-positive cells were defined as surrogates for NETs. Allophycocyanin (APC); Fluorescein isothiocyanate (FITC); Neutrophil extracellular traps (NETs); Kawasaki disease (KD); Myeloperoxidase (MPO)

Table 2. Comparison of H3cit detection data and clinical characteristics among three groups.

Characteristics	Acute KD	Sub-acute KD	HC	*P*
Male (n)	18	15	14	NS
Age at onset (m)	8.50±10.40	19..50±11.40	22.75±16.76	NS
CRP (mg/dl)	83.19±54.87	12.67±2.62	3.21±0.56	**
White blood cell count ($\times10^9$ /l)	22.00±5.58	10.52±2.11	4.65±0.81	**
Neutrophils (×109 /)	12.30±3.32	4.81±1.64	2.04±0.45	**
Platelet count ($\times10^9$ /l)	301.72±118.56	430.73±2.62	103.08±0.23	**
H3Cit Level	4.932 (2.1,9.6)	1.556 (1.3,2.5)	1.320 (1.1,3.0)	**

Citrullinated histone 3 (H3Cit); Kawasaki disease (KD); C-reactive protein (CRP); Healthy control (HC)

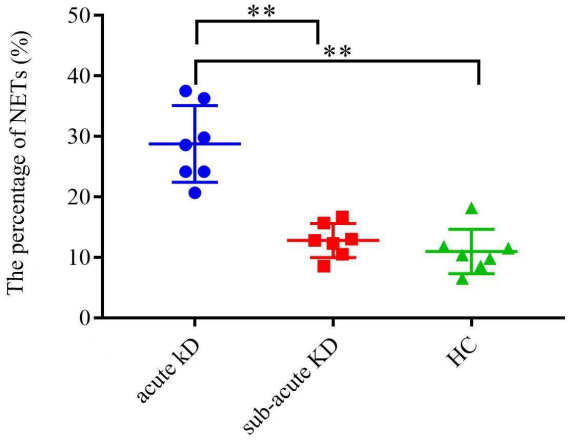

Fig. 2. Quantification of NETs in circulating NETs in samples from the healthy controls (n=7), acute KD patients (n=7), and sub-acute KD patients (n=7). Mann-Whitney test: $^{**}p<0.01$. Neutrophil extracellular traps (NETs); Kawasaki disease (KD); Citrullinated histone 3 (H3Cit); Healthy control (HC)

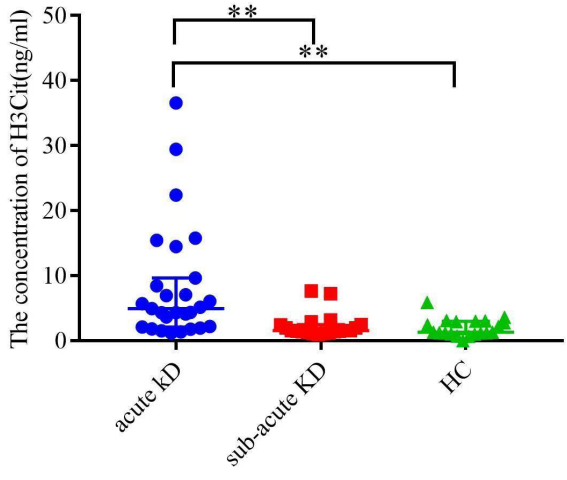

Fig. 3. ELISA-detection of plasma H3Cit levels in three groups. Mann-Whitney test: $^{**}p<0.01$. Kawasaki disease (KD); Healthy control (HC); Citrullinated histone 3 (H3Cit)

platelet counts.

The level of H3Cit significantly elevated in the acute KD group in comparison with that in the HC group (4.932(2.1,9.6) vs 1.320(1.1,3.0) ng/ml, p=0.000). Besides, IVIG treatment significantly decreased the H3Cit levels in KD patients (4.932(2.1,9.6) vs 1.556(1.3,2.5) ng/ml, p=0.000). There was no significant difference between HC and the sub-acute KD groups (1.556(1.3,2.5) vs 1.320(1.1,3.0)ng/ml, p=0.534, Fig. 3).

DISCUSSION

KD is associated with innate immune disorders. The levels of a variety of cytokines are elevated in KD patients (2). NETs take the central stage in driving autoimmunity. It increases TNF-α, IL-1β , and hypoxia-inducible factor-1α (HIF-1α) mRNA expression in humans (22). NETs can also activate other immune cells, such as T cells, B cells, and antigen-presenting cells (APCs). KD is also a thrombotic disease. Coronary artery aneurysm with thrombosis is the most dangerous factor among various complications of KD in the sub-acute and convalescent. Studies have shown that the levels of DNA histone complex and free DNA (cf-DNA), the key components of NETs, increased in a variety of thrombotic cardiovascular diseases, and as a sensitive biomarker, they positively correlated with the severity of the disease (23, 24). More and more studies have shown that NETs can promote coagulation. NETs have established a close relationship between inflammation and thrombosis. NETs can over-activate coagulation and lead to disseminated intravascular coagulation (25, 26). The main

interactions between platelets, coagulation system components, and NETs have been gradually clarified, forming a new concept called immunothrombosis. Therefore, we think that the immune response of KD may have a common immune mechanism with the production of NETs.

In this study, we quantified NETs generation in patients with KD by flow cytometry-based approach. The results showed that the NETs count in the acute KD group was significantly higher than that in the HC group, indicating that NETs' formation may be involved in the pathogenesis of KD. Yoshida et al. found that the serum levels of cfDNA measured by a cell death detection ELISA were significantly higher in the acute phase of KD than in the convalescent phase or HCs (27). These findings were consistent with our results.

The efficacy of IVIG therapy on patients with KD has been established, but the mechanism by which high-dose immunoglobulin ameliorates the vasculitis of KD remains obscure. It includes Fc-mediated inactivation of macrophages, consumption of activated complement proteins, neutralization of pathogenic autoantibodies by idiotype antibodies, and the correction of cytokine imbalance (28). IVIG may also influence the number and function of regulatory T cells (Tregs) which helps control inflammation.Various other effects of IVIG on platelet adhesion, oxidative stress, and neutrophil function in KD have also been reported (29). In this study, we found that after IVIG treatment, the level of NETs significantly decreased in KD patients, indicating that IVIG may reduce the immune response of KD by inhibiting the NETs formation. However, the precise mechanism by which IVIG suppresses NETs formation remains unknown. Okubo et al. recently demonstrated that IVIG-induced lactoferrin secretion, at least in part, can be involved in the IVIG-mediated inhibition of NETs formation (30-32). Further prospective clinical studies are needed to demonstrate the inhibitory effects of IVIG on NETs.

To our knowledge, the study of the serum H3Cit in children is very limited. Our findings demonstrate that the level of H3Cit significantly elevated in acute KD. H3Cit peptide has been proven to increase the permeability of human umbilical venular endothelial cells (HUVEC) in vitro. It is also reported that histones may directly lead to inflammatory injury through host tissue injury (33, 34). Increased levels of citrullinated histone H3 in patients with juvenile idiopathic arthritis (JIA) reported by Parackva could contribute to ongoing inflammation associated with autoimmunity, which can be ameliorated by regulating inflammation using immunotherapy (35). Therefore, we have a hypothesis that H3Cit may participate in vascular endothelial cell damage, increasing vascular permeability and leading to further damage of small and medium-sized blood vessels in KD. Certainly, this hypothesis needs to be verified by further biological and clinical experiments. Arginine deaminase type 4 (PAD4) is transferred to the nucleus after activation to induce citrullination of histone, leading to chromatin depolymerization, thus further increasing the production of NETs. Therefore, NETs and H3Cit have positive feedback interaction. In our study, we found that the level change of NETs and H3Cit was consistent.

Our study has its own limitations. Firstly, because of the high requirements for specimen preparation, the sample size of NETs is relatively small. Studies on NETs are challenging as neutrophils have a short life and become easily activated. In the second part of our research, we measured the level of H3Cit in serum samples as a supplement. Secondly, the data of the recovery period of KD are not included, and statistical associations might not necessarily indicate any cause-effect relationships. Finally, the clinical relevance of increased serum H3Cit in relation to KD course, or vascular outcomes remains to be established.

CONCLUSION

Acute KD is characterized by increased formation of NETs and high levels of H3Cit. IVIG may reduce the level of H3Cit in KD patients by preventing NETs information to achieve the therapeutic effect. These findings indicated that inhibiting H3Cit might be another potential therapeutic option for KD. Further studies on NETs and H3Cit will help improve our understanding of KD and accurate diagnosis, which remains challenging.

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AUTHORS' CONTRIBUTION

Jing Hu,We i Qian, Jingjing Ling, and Shiying Zuo: conceptualization, formal analysis. Shiyinng Zuo and Jingjing Ling: funding acquisition. Wei Qian, Tianhe Wang: methodology. Jing Hu and Wei Qian: writing-original draft. Jing Hu, Jingjing Ling, and Shiying Zuo: writing, review, and editing. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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