TSLP induces platelet mitophagy and promotes thrombosis in Kawasaki disease

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Research Article

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Abstract

Background

Kawasaki disease (KD) is an acute systemic vasculitis primarily affecting infants and children with an unclear etiology. Coronary artery aneurysm (CAA) is a common manifestation in severe KD patents, which may lead to thrombotic cardiovascular events such as heart attack and stroke even years after onset. We and others have previously reported systemic platelet activation in KD patients. Thymic stromal lymphopoietin (TSLP) is a recently identified interleukin-7 (IL-7) like cytokine associated with promoting pathological inflammation and most notably, platelet activation. The present study is to investigate the role of TSLP in KD-associated thrombosis.

Methods

To discover potential proteins underlying platelet activation in KD, we conducted a protein chip assay of 34 cytokines and discovered a significant upregulation in thymus stromal lymphopoietin (TSLP). ELISA corroborated the upregulation of TSLP in another group of KD patients. Clinical samples (plasma and platelets) from KD patients and healthy controls were analyzed via flow cytometry, immunofluorescent confocal microscopy, western blot, immunoprecipitation and thrombosis assays to reveal the underlying mechanisms.

Results

Among the 34 cytokines, we discovered several were aberrantly expressed, nine of which were continuously elevated after IVIG treatment and maintained during the convalescence in KD patients compared to healthy controls, including TSLP. The upregulation of TSLP in KD patients was confirmed by ELISA, which showed a further increase of TSLP in convalescent patients complicated with thrombosis. The expression of TSLP receptors (TSLPRs) on platelets were also significantly upregulated in KD patients complicated with thrombosis. Platelet activation, apoptosis, and mitochondrial autophagy (mitophagy) were increased in KD patients, which increase was exacerbated in convalescent patients complicated with thrombosis. In vitro, TSLP induced platelet activation and platelet mitophagy in healthy blood donors as we observed in KD patients. TSLP, similar to mitophagy agonist CCCP, promoted thrombosis, which was attenuated by the mitophagy inhibitor Mdivi-1. Co-immunoprecipitation in TSLP-treated platelets revealed TSLPR bound to mitophagy regulators, Parkin and VDAC1, suggesting a potential novel TSLP-mediated platelet mitophagy pathway.

Conclusions

Our results demonstrate that TSLP induces platelet mitophagy via a novel TSLPR/Parkin/VDAC1 mitophagy pathway that promotes thrombosis in KD. These results suggest TSLP as a novel therapeutic target against KD-associated thrombosis.

Introduction

Kawasaki disease (KD), also known as mucocutaneous lymph node syndrome, is a common pediatric vasculitis of medium and small muscular arteries that affects mainly children under 5 years old[1,2]. The main cause of death in KD is heart attack and stroke, which is often caused by occlusive thrombosis precipitated by coronary artery aneurysm (CAA)[3,4]. The risk of thrombosis persists up to years following initial onset and treatment. Indeed, our group and several others have reported elevated platelet activation in KD patients[5–7], which may serve as a risk factor for thrombosis in KD patients with pre-existing CAA[8,9]. The pathogenesis of platelet activation and thrombosis in KD remains elusive, although increased thrombocytosis[10], disordered platelet function[11], and increased platelet-derived microparticles[12] have been reported. It is imperative to uncover its pathophysiology, particularly the mechanism that causes platelet activation, to identify novel therapeutic targets against KD-associated thrombosis.

Three traditional antiplatelet pathways[13] are currently used for the clinical treatment of KD: thromboxane A2 pathway (such as aspirin), ADP pathway (such as clopidogrel), and platelet receptor pathway (such as abciximab)[14,15]. For KD patients with coronary artery aneurysm and thrombosis, combined treatment of antiplatelet and thrombolysis or anticoagulation therapy is usually used[1]. However, these processes cannot completely inhibit platelet activation and thrombosis, indicating that there may be other underlying platelet activation mechanisms regulated by unknown factors. Studies have reported that cytokines contribute to inflammation and thrombotic responses[16,17]. However, whether plasma cytokines contribute to platelet activation and thrombosis in KD is unknown.

Thymic stromal lymphopoietin (TSLP) is an IL-7 like cytokine that is mainly derived from epithelial cells, fibroblasts and mast cells[18]. Accumulating evidence implicates the dysregulated expression of TSLP in multiple diseases such as asthma, allergic rhinitis, leukemia and atherosclerosis[19–22]. Recently, the roles of TSLP and TSLPR on platelets have been linked to platelet activation and thrombus formation through PI3K/Akt signaling[23,24]. However, the role of TSLP in aberrant platelet activation and thrombosis in KD has not been previously reported.

Platelet autophagy is involved in the regulation of platelet activity[25,26]. Not surprisingly, mitophagy, a selective autophagy that regulates mitochondrial quality also occurs in platelets and is emerging as an important regulatory mechanism in platelets. Since the mitochondrion is essential for energy production, it is no surprise that enhanced mitophagy is linked with platelet autophagy and activation. Recent reports[27] demonstrate platelet mitophagy promotes thrombosis, however, the role of platelet mitophagy in KD platelet activation and thrombosis has never been explored.

In the present study, we discovered that TSLP is significantly upregulated in KD and promotes platelet mitophagy and thrombosis via a novel TSLPR/Parkin/VDAC1-dependent signaling pathway. Our findings further elucidate the mechanism of thrombosis in KD and identify TSLP as a potential novel anti-thrombotic therapeutic target.

Materials And Methods

Study objects

Samples of KD patients and age-matched healthy controls (HC) were collected from the Guangzhou Women and Children Medical Center in China, between July 2016 and March 2021(Supplemental Materials Table I). All specimens were stored in the clinical biological resource bank (Clinical Bio-bank) of this hospital. KD patients were diagnosed by our hospital cardiology physician, according to the latest version of the American Heart Association's 2017 revised diagnostic criteria and treatment guidelines[1]. Coronary artery abnormalities of KD patients were categorized according to their Z score: no coronary artery damage(Z < 2 mm), coronary artery dilation(Z > 2 ~ < 2.5mm), large CAA (≥ 8.0 mm or Z ≥ 10 mm), medium CAA (< 8.0 mm and Z ≥ 5 ~ < 10 mm), and small CAA (Z ≥ 2.5 ~ < 5 mm). This study was approved by the Ethics Committee of Guangzhou Women and Children's Medical Center (Number: 2014073009 and 2018052105). All participants' parents/guardians gave written informed consent in accordance with the Declaration of Helsinki. We categorized blood samples from KD patient samples into either of three stages, the acute phase (patients before Intravenous IgG (IVIG) and after IVIG treatment within 10 days), subacute phase (after treatment 11 days-1 mouth) and convalescence phase (Aspirin or other antiplatelet drug treatment more than 1–6 mouths).

Preparation of human platelets

Venous blood was drawn from healthy controls and KD patients then collect with sodium citrate or ACD (2-4mL) anticoagulant tube, and pretreated with 75nM prostaglandin E1 (PGE1; Catalog No. : HY-B0131, MEC) to prevent platelet activation. Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 900 rpm at 22˚C for 10mins, platelet-poor plasma (PPP) was obtained by centrifugation of PRP at 3500 rpm at 22˚C for 10mins, and platelet pellets at the bottom of the tube[28,29]. Platelet counts in PRP were performed with an automatic blood cell analyzer (sysmex XS-500i). To prepare washed platelets, platelet pellets were gently washed twice with CGS buffer (0.123 M NaCl, 0.033 M D-glucose, 0.013 M trisodium citrate, pH 6.5) containing 75nM PGE1, then the washed platelets were suspended in modified Tyrode's buffer (MTB; 2.5 mM Hepes, 150 mM NaCl, 2.5 mM KCl, 12 mM $NaHCO_3$, 5.5 mM D-glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) containing 75nM PGE1 at 3–5 × 10^8 platelets/mL, and incubated at room temperature for 1 h before use.

Regents and antibodies

Bafilomycin A1 (Baf-A1), mitochondrial division inhibitor-1 (Mdivi-1) and mitophagy agonist Carbonyl cyanide m-chlorophenyl hydrazone, Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were purchased

from Selleckchem. Prostaglandin E1 (PGE1) was purchased from MCE. Recombinant human TSLP was purchased from Beyotime and peprotech. $CaCl_2$ (0.025M) and thrombin were purchased from Diagnostica Stago. Antibodies of β-actin, TSLP, LC3A/B, Bcl-xL, Bax, IgG, and Caspase-3 were purchased from Cell Signaling Technology. TSLPR, Parkin, and PINK1 antibodies were obtained from Abcam. Antibodies of TSLP receptors (TSLPR and IL-7R), VDAC1, Tom20 and protein A/G PLUS-Agarose immunoprecipitation reagents were purchased from Santa Cruz Biotechnology. The anti-CD41a and anti-CD62p were purchased from eBioscience. Human TSLP ELISA kit was purchased from R&D Systems, soluble CD40L (sCD40L) and soluble P-selectin (sP-selectin) ELISA kits were purchased from MultiSciences. MitoTracker antibody and MitoProbe™ TMRM Assay Kit were purchased from Thermofisher. Annexin V-FITC/PI Apoptosis Detection Kit was purchased from Keygen Biotech.

Multiplex Analysis and Quantification of Cytokines

The Bio-Plex Pro Human Inflammation Group 34-Plex 171AL001M (Bio-Rad, Hercules, CA) kit was used to detect the cytokine content in plasma samples selecting by “Clinical Bio-bank” from KD patients and healthy control. The protocol was followed according to the manufacturer’s instructions. The Bio-Plex Verification Kit Version 3.0 (Bio-plex200, Bio-Rad, Hercules, CA) was used to verify the hydrodynamic performance, consistent optical alignment, double identification and identification of single bead signatures. The measurement was performed using the Bio-Plex protein array system integrated with Bio-Plex Manager software version 3.0 (Bio-Rad, Hercules, CA).

ELISA

ELISA was used to detect plasma concentrations of TSLP (R&D Systems), sCD40L and sP-selectin (MultiSciences). ELISA experiments were carried out according to the manufacturer's protocol as we previously described[5]. Briefly, PPP samples (40–100μL) were added into each well of a microtiter plate followed by 10–100μL of the detection antibody. Following 1-2h incubation at 37˚C, 50–100μL of streptavidin-HRP was added to each well and allowed to incubate for 30-60mins. The wells were subsequently washed and 50–100μL color reagent A and 50–100μL color Reagent B were added, gently vortexed, and incubated at 37˚C for 10 minutes in the dark. The reaction was quenched by adding 50μL Reagent C and plates were read at 450 nm with a Variskan Flash (Thermo Scientific).

Measurement of platelet activation, mitochondrial membrane potential (ΔΨm), PS exposure by flow cytometry

To assess platelet activation, PRP was prepared from human whole blood as described above. PRP (3–5μL) was labelled with-anti-CD41a and anti-CD62p, incubated at room temperature for 15-30mins. The reaction was stopped by adding PBS, and then analyzed by flow cytometry analysis (BD FACScanto II). Platelets were identified and gated by their characteristic forward and side scatter properties. A total of 10,000 platelets were measured from each sample as we previously described[30,31].

Platelets were pelleted from PRP and subsequently resuspended in MTB adjusted to a concentration of $3–5\times10^8$ platelets/mL. Platelets were incubated with 1μM tetramethylrhodamine methyl ester (TMRM) at 37˚C for 30 mins. Platelets were subsequently incubated with 5μL of Annexin V at room temperature for 15 mins. Flow cytometry was then used to analyze ΔΨm and PS externalization[32–34]. Platelets were identified and gated by their characteristic forward and side scatter properties. A total of 10,000 platelets were analyzed from each sample.

Observation of autophagosomes by transmission electron microscopy

The platelet pellet was prepared as above described, then washed twice and fixed with 2.5% glutaraldehyde solution and 1% osmium tetroxide solution. Finally, the platelets were dehydrated with an ethanol solution then embedded and cut into ultrathin 100 nm sections. After staining with a saturated uranyl acetate solution and a lead acetate solution, the platelets were observed and photographed under a transmission electron microscope.

Immunofluorescence and confocal microscopy

PRP was prepared from human whole blood as described above. MitoTracker® was incubated with platelets (100μL of PRP diluted in 1mL MTB) at room temperature for 15mins. Platelets were then pelleted by centrifugation at 3500 rpm for 10mins at 25˚C, resuspended in MTB (400μL) by centrifuged again at 7200 rpm for 3mins to adhere to the glass slide. Platelets were then fixed with 4% paraformaldehyde (100–200μL) for 15mins, washed three times with PBS, permeabilized for 10mins in 0.25% Triton X-100, washed 3 times with PBS and then blocked (5% BSA, 0.1% Triton X-100 in PBS) for 30mins at room temperature. After the sealing solution was removed, the platelets were incubated with a primary antibody against LC3, TSLPR (1% BSA in PBS 1:200) at 4˚C overnight. Next, a fluorescent secondary antibody (1% BSA in PBS 1:1000) was added to platelets and incubated for 2h at room temperature in the dark, then washed 3 times with PBS. After platelets were sealed with anti-fluorescence quenching liquid, the stained platelets were observed using a Zeiss LSM 800 confocal microscope with 63x oil immersion lens and photographed as we previously described[35].

Western blotting analysis

Platelets from patients and healthy volunteers were obtained by gradient centrifugation. Healthy volunteers PRP were treated with recombinant human TSLP (200 ng/mL or 500 ng/mL) for 3h, and then lysed with an equal volume of lysis buffer on ice for 30mins. The protein concentration was determined using a BCA protein assay kit (Applygen Beijing) according to the manufacturer's protocol. Each sample was loaded in equal proportion to a 10%-12% SDS gel and separated via polyacrylamide gel electrophoresis (PAGE) and transferred to a PVDF membrane. Either TSLP, TSLPR, Caspase-3, Bax, Bcl-xL, PINK1, Parkin, LC3A/B, Tom20, β-actin, VDAC1 or IL-7R (1:2000) antibody was incubated with the membrane at 4˚C overnight, then washed and incubated with the corresponding secondary antibody for

2h at room temperature. Antibody binding was detected with the ECL detection reagent by Amersham image 600 Software and the bands were quantified with Quantity Image J as we previously described[36].

Immunoprecipitation analysis

PRP was pretreated with or without Baf-A1 (400nM for 1h) and then treated with recombinant human TSLP (500 ng/mL for 3h). Platelets were then lysed and mixed with the specific target antibody for Parkin IP (1:50 dilution); VDAC1 IP (1:30 dilution) or TSLPR IP (1:30 dilution), and the IgG control group as a negative control, and incubated for 1h at 4˚C. Then, 20μL of resuspended A/G PLUS-Agarose was added to each sample and incubated at 4˚C on a rocker platform overnight. Following this incubation, samples were pelleted and washed 4 times before being loaded on SDS-PAGE. The subsequent steps were according to the aforementioned western blot protocol[37].

In vitro thrombosis and clot retraction assays

1) Whole blood thrombosis assay

Glass tubes were coated with Sigmacote® and dried overnight. In one experiment, 200μL aliquots of PRP were incubated with a gradient concentration of recombinant human TSLP (0, 100, 200 or 500ng/mL) in 37˚C for 3h using 1.5 NIH unit/mL thrombin as a positive control. In another experiment, 200μL aliquots of PRP were incubated with CCCP (10μM) or Mdivi-1 (10μM) treatment for 1h followed by incubation with recombinant human TSLP for 3h. PRP samples were then diluted with 200μL PBS, 50μL whole blood and 100μL $CaCl_2$ (0.025M) incubated at 37˚C with images photographed at 30min/1h/1h30m/2h[38].

2) Platelet-rich plasma clot retraction assay

As described for whole blood thrombosis without the addition of whole blood. Each condition received 200μL PRP, 200μL PBS and 100μL $CaCl_2$ (0.025M) and was left to clot in 37˚C, with clot retraction photographed over time[39].

Statistical analyses

All experiments were repeated at least 3 times, the data are presented as the mean ± SD. Differences between the experimental groups were assessed by a two-tailed unpaired t-test, and in datasets with 3 or more groups was performed by one-way analysis of variance followed by Tukey's test Differences were analyzed by GraphPad Prism 7 with *p* value less than 0.05 considered as statistically significant.

Results

KD patients with thrombosis showed increased platelet activation and apoptosis

We previously reported that plasma levels of soluble CD40L (sCD40L) and soluble P-selectin (sP-selectin) were elevated in KD patients, and positively correlates with the degree of coronary artery damage in Kawasaki disease patients[5]. In the present study, we examined plasma from another group of KD convalescent patients and found a significant increase in the platelet activation marker P-selectin (CD62p) (Fig. 1A**)**. Similarly, sP-selectin and sCD40L were significantly elevated in the plasma of acute (patients before IVIG and after IVIG) and convalescent patients relative to healthy controls (Fig. 1B**&C**) and significantly elevated in convalescent patients complicated with thrombosis relative to those without (Fig. 1D**&E**). These findings confirmed that baseline platelet activation is significantly greater in acute and convalescent KD patients, which is exacerbated in patients complicated with thrombosis.

Previous reports have shown that platelet activation is associated with platelet mitochondrial dysfunction and control of the platelet life span[40]. When dysfunction of platelet mitochondria occurs, the mitochondrial membrane potential will decrease, the apoptosis protein will change, PS valgus, and calcium ion changes, which will eventually lead to platelet apoptosis[32,33]. Thus, we analyzed apoptotic signaling and mitochondrial dysfunction in platelets from KD patients. Using flow cytometry, we found a significant reduction in mitochondrial membrane potential (Δψ m) in platelets from KD patients relative to healthy controls (Fig. 2A). Western blot analysis confirmed an upregulation in hallmark platelet apoptotic proteins including Bax, Caspase-3 and VDAC1, and the downregulation of the anti-apoptotic protein Bcl-xL. Notably, apoptotic markers were most enhanced in convalescent patients complicated with thrombosis **(**Fig. 2B**)**. Consistent with these findings, flow cytometry analysis revealed a significant increase in the percentage of Annexin V-positive platelets in the KD convalescent patients compared to healthy controls (Fig. 2C). Taken together, the above data suggests an elevation in both platelet activation and apoptosis in KD patients, especially in those complicated with thrombosis.

Platelet autophagy is upregulated in KD

Since we found increased apoptosis in KD patients, we next decided to investigate whether basal platelet autophagy was affected. Fluorescent confocal microscopy revealed that the platelet autophagy marker LC-3 was significantly increased in platelets from KD patients relative to healthy controls (Fig. 3A). Electron microscopy also found an increase in the number of platelet autolysosomes in KD patient platelets (Fig. 3B). Consistent with these data, western blot analysis showed an increase in the autophagy proteins PINK1, Parkin, Tom20 and LC3A/B in KD patients, especially in patients with thrombosis (Fig. 3C). Collectively, these data confirm platelet autophagy is upregulated in patients with KD and is closely related to KD-related thrombosis.

Plasma TSLP and platelet TSLP receptors were elevated in KD patients complicated with thrombosis

Given the inflammatory nature of KD, we suspected dysregulated cytokines may contribute to platelet activation. To investigate this, we utilized a protein chip assay to quantify the expression of 34 cytokines in plasma from KD patients in acute, subacute and recovery phase comparison with healthy controls and febrile controls. Several were aberrantly expressed, nine of which were continuously elevated after

treatment and maintained during the recovery phase in KD compared to healthy controls, including TSLP (Fig. 4A**&B**). Since TSLP/TSLPR has previously been linked to platelet activation and thrombosis[23], we decided to narrow our focus on this protein. We divided the patients into two groups according to echocardiography, KD with thrombosis and those without thrombosis (Fig. 4C). ELISA analysis corroborated the upregulation of plasma TSLP in acute (before IVIG and after IVIG) and convalescent phase KD patients in different group of samples (Fig. 4D), which also showed a significant increase in convalescent KD patients complicated with thrombosis relative to those without (Fig. 4D). Similarly, the expression of the TSLP receptors (TSLPR and IL-7R) on platelets was upregulated in KD patients, especially in patients with thrombosis (Fig. 4E).

TSLP promotes mitophagy-mediated platelet activation and apoptosis causing thrombosis in vitro

We next investigated the effect of TSLP on platelet activation and mitophagy in vitro. TSLP treatment on healthy human platelets caused a significant increase in CD62p (Fig. 5A) and dose-dependently accelerated human PRP and whole blood thrombosis **(**Fig. 5B**)**. To investigate TSLP in mitophagy, fluorescent confocal microscopy was used to image the mitophagy marker LC-3 and found a significant increase in platelets following TSLP treatment **(**Fig. 5C**)**. Western blot analysis also revealed that platelet mitophagy and apoptotic proteins were increased by TSLP treatment in a dose-dependent manner **(**Fig. 5D**)**. Based on this apparent link with mitophagy, we used TSLP in combination or in comparison to the mitophagy agonist (CCCP) and mitophagy inhibitor (Mdivi-1) in a thrombosis assay. Interestingly, CCCP showed similar thrombosis to TSLP, whereas Mdivi-1 alleviated thrombosis induced by TSLP (Fig. 5E). Taken together, these data suggest that TSLP induces platelet mitophagy causing downstream platelet activation and thrombosis.

TSLPR binds Parkin and VDAC1 to trigger TSLP induced platelet mitophagy

Multiple studies have demonstrated that the PINK1/Parkin signalling pathway is involved in the regulation of mitophagy[41–43]. Thus, we explored whether TSLP regulates KD platelet mitophagy through the PINK1/Parkin signaling pathway. In vitro, TSLP dose-dependently increased the expression of TSLPR and IL-7R in platelets **(**Fig. 6A**)**. TSLP also significantly increased the expression of PINK1, Parkin and VDAC1 in platelets (Fig. 6A**&B**). Furthermore, pretreatment with the late-stage autophagy inhibitor Baf-A1 did not prevent TSLP-induced elevation of PINK1, Parkin, VDAC1 and TSLPR (Fig. 6B). Together, these results suggest that TSLP may promote platelet mitophagy through the PINK1/Parkin pathway.

Based on a study that showed TSLP may cause TSLPR internalization[44], we hypothesized that TSLP caused TSLPR internalization and translocation to the mitochondria where it directly promotes mitophagy. Indeed, immunoprecipitation (IP) results show that Parkin and VDAC1 both independently bound TSLPR in platelets and their binding to each other and TSLPR was significantly enhanced following TSLP treatment (Fig. 6C**&D**). This result is consistent with previous reports[45], in which Parkin

complexes with VDAC1 to induce mitophagy. Further evidence for the TSLPR-Parkin-VDAC1 interaction comes from an additional IP using TSLPR to pull down Parkin and VDAC1 (Fig. 6E), effectively demonstrating a triple co-IP (Fig. 6C-E). Immunofluorescent confocal imaging confirmed TSLPR upregulation and colocalization to the mitochondria in platelets following TSLP-treatment (Fig. 6F). Taken together, our results show that TSLP induces platelet mitophagy through the TSLPR binding with Parkin and VDAC1.

Discussion

In the current study, we describe a novel mechanism of platelet activation and thrombosis induced by TSLP in KD. Using clinical samples, we found significantly upregulated expression of plasma TSLP in KD patients relative to healthy controls, which was exacerbated in patients complicated with thrombosis. Furthermore, TSLP receptor (TSLPR and IL-7R) expression was significantly enhanced on platelets of KD patients complicated with thrombosis. Interestingly, we found increased platelet mitophagy and apoptosis in KD patients complicated with thrombosis, which TSLP induced in vitro. Lastly, TSLPR bound to mitophagy regulators Parkin and VDAC1 respectively following TSLP treatment, suggesting a novel TSLP-mediated mitophagy pathway in platelets. Taken together, our findings uncover a novel mechanism of platelet activation and thrombosis in KD and suggest TSLP as a novel anti-thrombotic target.

Our work identifies that upregulated TSLP expression at least partially underlies platelet activation and thrombosis in KD. TSLP-induced platelet mitophagy and activation likely promotes thrombosis by promoting platelets to directly bind its ligands mediating platelet aggregation[46–48], and to harbor phosphatidylserine (PS) on its surface, which promotes a hypercoagulable state through cell-based thrombin generation[34,49]. Current anti-thrombotic therapies may not effectively inhibit TSLP-induced thrombosis in KD given its unique mechanism, which may explain the persistent platelet activation and thrombosis in treated patients. A previous report identified that TSLP activates platelets through the PI3K/AKT pathway. However, since we found that the mitophagy inhibitor Mdivi-1 significantly attenuated TSLP-mediated in vitro thrombosis, we propose that its effect on PI3K/AKT signaling may be downstream of its induction of mitophagy[23,50].

Mitophagy is mainly regulated by the PINK1/Parkin signaling pathway[51]. Recombinant Voltage Dependent Anion Channel Protein 1(VDAC1) is a critical substrate of Parkin responsible for the regulation of mitophagy and apoptosis[52]. Interestingly, we found that TSLPR bound to Parkin and VDAC1 and TSLP treatment enhanced the independent binding of TSLPR/Parkin/VDAC1. TSLP binding TSLPR is reported to cause receptor internalization[44], which is consistent with our findings of TSLPR co-localization with the mitochondria in platelets following TSLP treatment. This TSLPR/Parkin/VDAC1 protein complex may be an important driver of TSLP-mediated platelet mitophagy in KD.

Several reports suggest platelet activation may be a major driver of inflammation in KD[8,53]. Platelets contain and release several proinflammatory cytokines upon activation such as TNF-α, IFN-β, and IL-6 that may propagate an inflammatory state[40,54]. Activated platelets also express P-selectin, which

promotes the formation of platelet-leukocyte aggregates, an important contributor in the progression of KD[55,56]. Inflammatory responses, platelet activation and thrombosis are inextricably linked. We propose that in addition to its role in thrombosis, TSLP may also contribute to systemic inflammation through its activation of platelets and release of pro-inflammatory factors. Recent studies have shown that TSLP upregulates inflammatory responses by inducing autophagy in T cells, which partially validating our hypothesis[57].

To our knowledge, we are the first to report evidence of increased platelet apoptosis in KD. It is interesting to note that the therapeutic mechanism of IVIG in KD is unclear, yet has previously been reported to inhibit platelet apoptosis in immune thrombocytopenia (ITP)[58]. Thus, it is conceivable that IVIG partially exerts its therapeutic effect in KD through its inhibition of platelet apoptosis. The cause of platelet apoptosis in KD remains unclear, however, considering the intimate link of platelet apoptosis and mitophagy, TSLP likely has a faciliatory role.

The origin of TSLP in KD remains unclear. A variety of stimuli and cytokines (IL-4, IL-13, IL-5, NF-κB, and TNF-α etc.) can activate TSLP production[59]. Interestingly, a recent report found that TSLP production in human dermal microvascular endothelial cells is also triggered by activated platelets in an IL-1β dependent manner[60]. Based on this report, TSLP may activate platelets, which triggers endothelial cells to produce TSLP creating a positive feedback cycle that drives TSLP production and platelet activation. Thus, TSLP neutralization may effectively normalize its plasma concentration and reduce platelets activation.

Many experts suggest targeting TSLP-mediated signaling as a novel therapeutic strategy against allergic diseases to neutralize its inflammatory function[61]. Interestingly, KD disease is marked by a persistent inflammatory state over many months that share several features to allergic disease inflammation including abnormal type 2 inflammation, Th17/Treg imbalance, and other immunopathogenesis[62]. In addition, anti-TSLP monoclonal antibodies are already used for the treatment of severe asthma, such as Tezepelumab and CSJ117[63]. Thus, TSLP may serve as a novel therapeutic target that effectively treats the inflammatory and thrombotic risks associated with KD.

A limitation of our research is that we did not use the TSLPR knockout KD mouse model to verify our results. However, our study utilized a large number of rare human clinical specimens, including convalesce stage KD patients with thrombosis, to identify the pivotal role of TSLP in KD.

Our results demonstrate a close relationship between TSLP and thrombosis in vivo and in vitro. TSLP induced platelet mitophagy and activation via the TSLPR/Parkin/VDAC1 signaling pathway to promote thrombosis in KD. Our findings highlight TSLP as an important contributor and novel therauptic target for KD-associated thrombosis.

Abbreviations

KD
Kawasaki disease
TSLP
Thymus stromal lymphopoietin
P TSLPR
Thymus stromal lymphopoietin receptor
INK1/Parkin
PTEN induced putative kinase 1/Parkin
VDAC1
Recombinant Voltage Dependent Anion Channel Protein 1
PS
Phosphatidylserine
IVIG
Intravenous IgG
CAA
Coronary artery aneurysm
Baf-A1
Bafilomycin A1
Mdivi-1
Mitochondrial division inhibitor-1
CCCP
Mitophagy agonist Carbonyl cyanide m-chlorophenyl hydrazone, Carbonyl cyanide 3-chlorophenylhydrazone
PGE1
Prostaglandin E1
PRP
Platelet-rich plasma
PPP
platelet-poor plasma.

Declarations

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Conflicts of Interest Statement

The authors report no conflicts of interest.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request. E-mail: guxiaoqiong@gwcmc.org.

Author contributions

All authors contributed significantly to this work. L.Y.F, D.T.M, and Q.G designed the study, performed most of the experiments, analyzed data, interpreted results, and wrote the manuscript. H.Y.Y performed Flow cytometer experiments, analyzed data, and interpreted results. D.C and C.N.C performed immunofluorescence and confocal microscopic analyses. L.P, Y.F.X, H.Z.Z, L.Z, Z.Y.J and W.Z.P provided project resources. L.Y.F, K.N.C performed immunoprecipitation experiments, analyzed data, and interpreted results and Picture editing. E.C and H.Y.Y performed article revision and language polish. G.X.Q and H.Y.N designed and supervised the study, interpreted results, and wrote the manuscript. All authors reviewed the manuscript. In addition, all authors have read and approved the manuscript.

Ethics statement

This study was approved by the Medical Ethics Committee of Guangzhou Women and Children's Medical Center (2014073009 and 2018052105) and was conducted according to the International Ethical Guidelines for Research Involving Human Subjects stated in the Declaration of Helsinki. Informed written consent was obtained from the guardians of the patients and controls.

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Figures

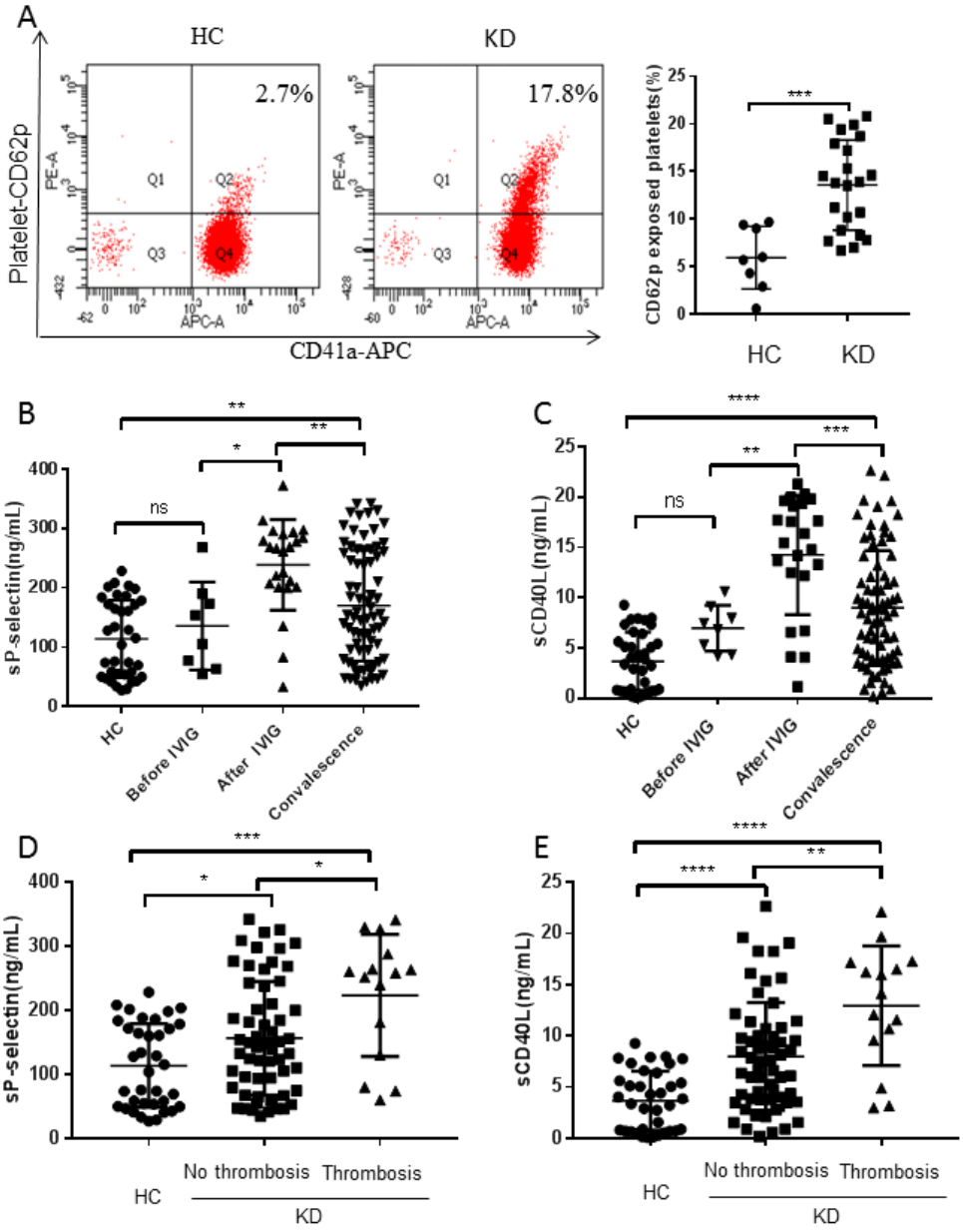

Figure 1

Platelet activation makers were elevated in KD patients with thrombosis. A: Flow cytometry was used to detect platelet activation marker CD62p and to analyze the percentage of CD62p positive platelets in HC (n=8) vs KD (n=22) (HC vs KD ***P =0.0004). **B-C:** ELISA detection of soluble P-selectin (sP-selectin) and soluble CD40L (sCD40L), respectively in the plasma of acute(Before IVIG n=8 and After IVIG n=23) and convalescent phase (n=75) patients compared with HCs (n=36) (HC vs KD/Convalescence **P=0.004

and****P<0.0001; After IVIG vs KD/Convalescence **P=0.007 and****P=0.0001) , **D-E:** ELISA detection of sP-selectin and sCD40L in the plasma of convalescent patients with or without thrombosis compared to healthy controls (HC vs KD/No thrombosis (n=60), *P=0.048 and ****P<0.0001; KD/No thrombosis vs KD/Thrombosis (n=15) *P=0.018 and **P=0.001). Notes: HC: healthy controls; KD: Kawasaki disease; KD/Thrombosis: Convalescent KD patients with thrombosis; KD/No thrombosis: Convalescent KD patients without thrombosis. *P<0.05, **P< 0.01, ***P< 0.001, **** P<0.0001 using t tests (nonparametric tests) and one-way ANOVA (multiple comparisons) test to analyze in datasets with three groups. The graph represents data from at least three replicates. All data are presented as mean ± SD.

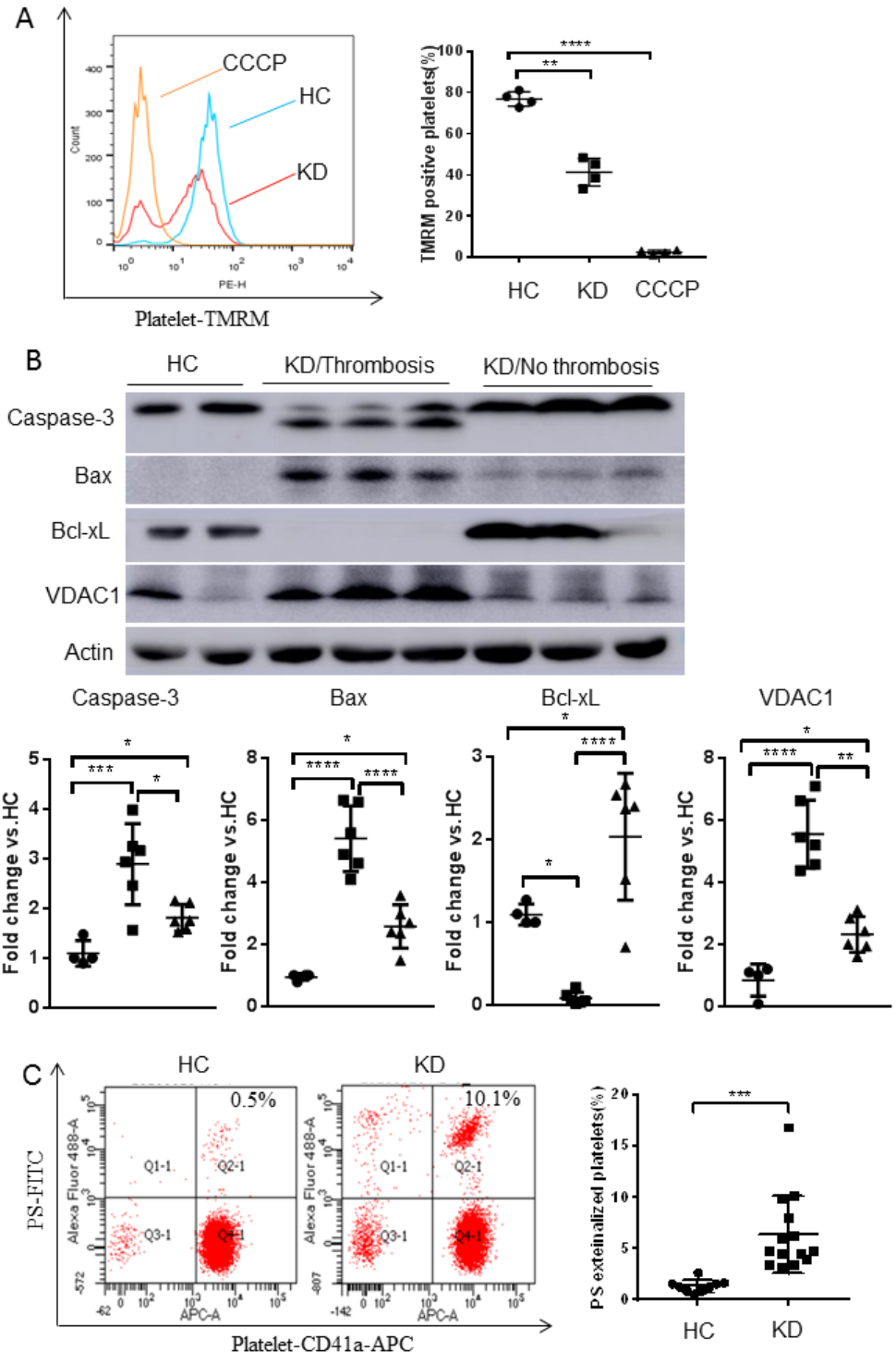

Figure 2

Impaired mitochondrial function in KD patients. A: TMRM positive platelets (Ψm) were measured by flow cytometry in HC (n =5) or KD (n = 10) with CCCP (5μL) as a positive control for mitophagy (HC vs KD ***P* =0.006). **B:** Western blot analysis of the expression of apoptosis-related proteins in platelets of each group. Quantification of HC (n=4) and KD with thrombosis (n=6) and without thrombosis (n=6) in platelets. **C:** Flow cytometry analysis and quantification showing the percentage of Annexin-V positive

platelets in HC (n = 9) and KD (n = 14) patient (HC vs KD ***P=0.001). *P<0.05, **P< 0.01, ***P< 0.001, **** P<0.0001 using t tests (nonparametric tests). Notes: ns: not significant. The graph represents data from at least three replicates. All data are presented as mean ± SD.

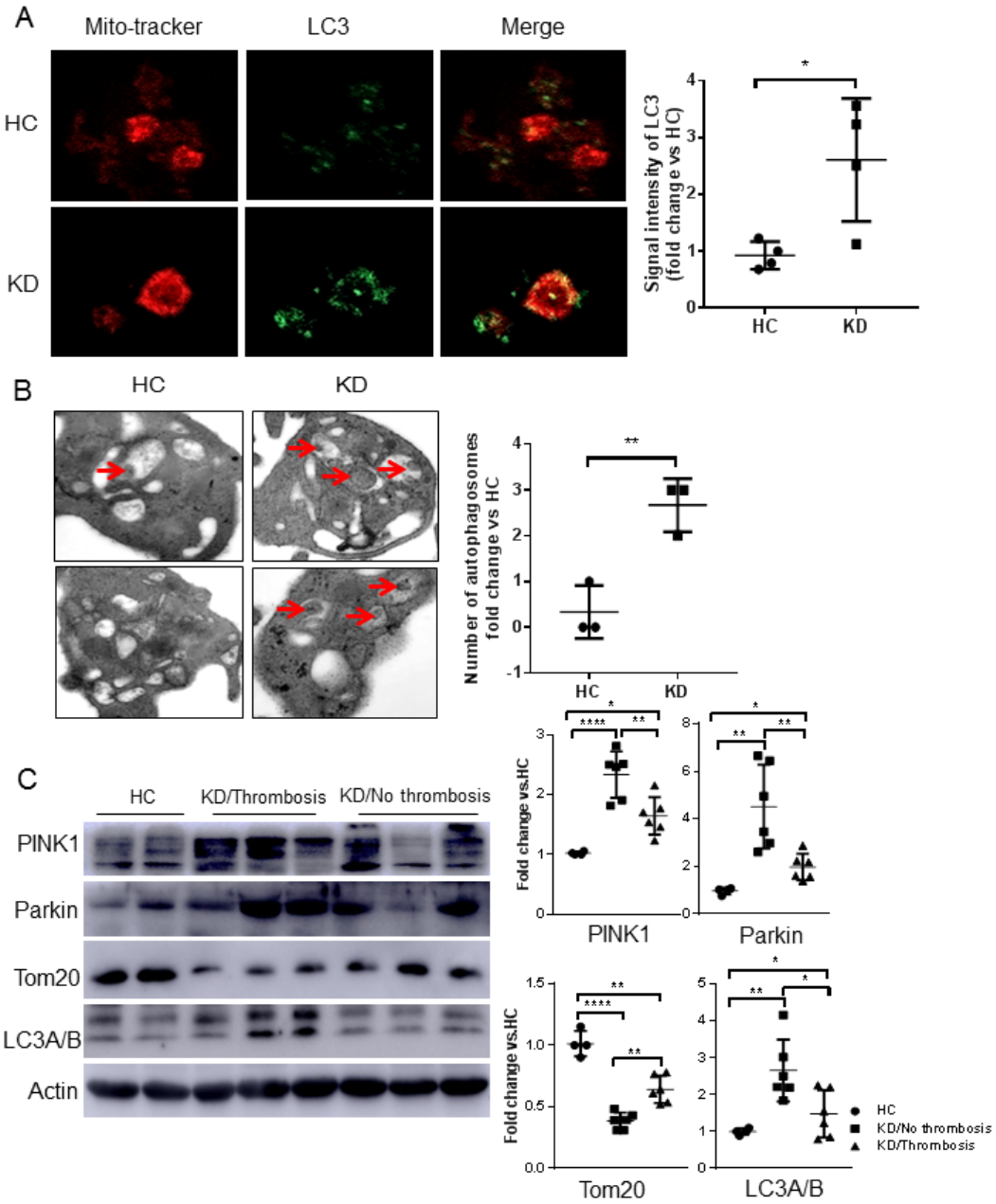

Figure 3

Platelet autophagy in KD was greater in those with thrombosis compared to those without thrombosis. A: Confocal microscopy was used to observe the fluorescence intensity of the autophagy marker LC3 in KD patients (n=4) compared with HCs (n=4) (HC vs KD $*P=0.023$). **B:** Morphological alterations in platelets (KD and HC group n=3) were detected by transmission electron microscopy (the red arrow indicates autophagosomes) (HC vs KD $**P=0.001$). **C:** Western blot analysis of the expression of autophagy-related proteins in platelets of each group, and quantification of the expression of HC (n=4) and KD with thrombosis (n=6) and without thrombosis (n=6) in platelets. $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$ using t tests (nonparametric tests) and one-way ANOVA (multiple comparisons) to analyze datasets with three groups. Actin served as a loading control. The graph represents data from at least three replicates. All data are presented as mean ± SD.

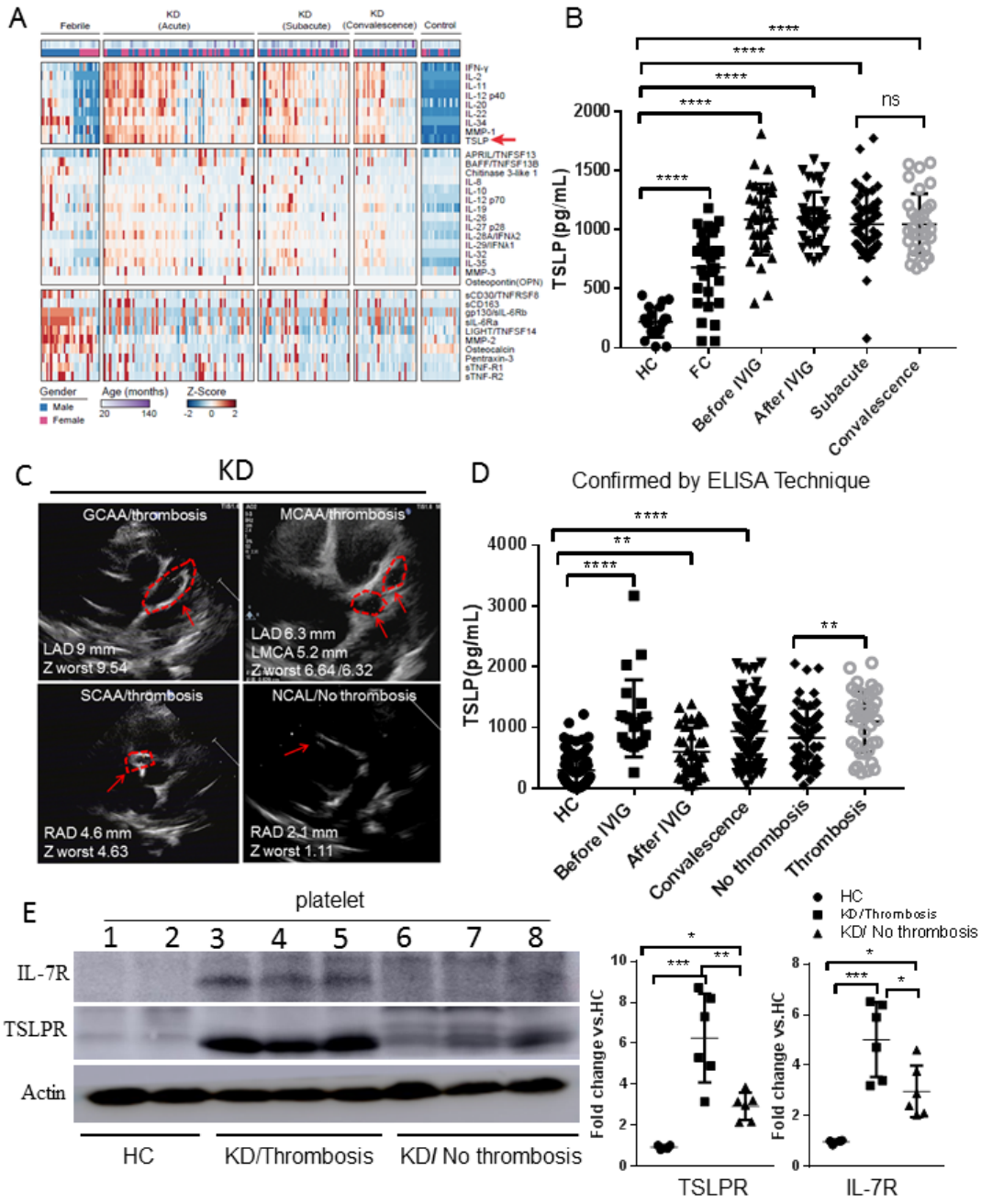

Figure 4

Expression of Plasma TSLP and platelet TSLP receptors increased in KD patients complicated with thrombosis. A: Heatmap of 34 differentially expressed inflammatory factors in plasma from healthy control (HC) (n=20), Febrile illness (n=30), KD patients in acute (n=74), subacute (n=47) and convalescence (n=32).**B:** Protein chip assay was used to detect plasma TSLP inflammatory factors of KD patients in the acute (Before IVIG n=35 and After IVIG n=39), subacute and convalescent (n=32) patients

relative to HCs (n=20) (all****P < 0.0001). **C:** KD patients were divided into four groups, small CAA (SCAA), medium CAA (MCAA)and giant CAA (GCAA) combined thrombosis and normal coronary artery (NCAL) without thrombosis according to coronary artery Z-worst. The dashed red circle is a coronary aneurysm, and the position indicated by the arrow is the coronary artery. Representative echocardiogram images of corresponding patients with KD/Thrombosis and KD/No thrombosis were shown. **D:** Verified the expression of TSLP in the plasma of another group of KD patients by ELISA in the acute (Before IVIG n=22 and After IVIG n=39) and convalescent (n=95) patients relative to HCs (n=63) (all****P < 0.0001), Convalescent KD patients with thrombosis (n=38) compared with without thrombosis (n=57) (KD/No thrombosis vs KD/Thrombosis **P =0.005).**E:** Western blot analysis and quantification of TSLP receptor (TSLPR and IL-7R) in platelets of HCs (n=4), KD with thrombosis (n=6), and KD without thrombosis (n=6). *P <0.05, **P < 0.01 using t tests (and nonparametric tests). Notes: HC: healthy control, KD/Thrombosis: Convalescent KD patients with thrombosis; KD/No thrombosis: Convalescent KD patients without thrombosis. *P <0.05, **P < 0.01, ***P < 0.001, **** P<0.0001 using one-way ANOVA (multiple comparisons) to analyze in datasets with three groups. The graph represents data from at least three replicates. All data are presented as mean ± SD.

Figure 5

TSLP promotes thrombus formation in vitro by promoting mitochondrial autophagy and activates platelets. A: Human platelets were stimulated with TSLP (500ng/mL) and the platelet activation marker CD62p was detected by flow cytometry (control vs TSLP *P=0.014).**B:** Human platelet-rich plasma (PRP) was stimulated with different concentrations of TSLP (100, 200 and 500ng/mL) for 3h while clot retraction was periodically photographed. Thrombin (1.5 NIH unit/mL)-treated PRP served as positive control**. C:** Human platelets were treated with TSLP, and the fluorescence intensity of autophagy marker LC3 protein was observed under confocal microscopy (control vs TSLP **P=0.006). **D:** Western blot analysis and quantification of the expression of mitophagy and apoptosis-related proteins in platelets after TSLP (200ng/ mL and 500ng/ mL) treatment for 3h. **E:** Thrombosis assay was compared after pretreatment with an autophagy agonist (CCCP 10μm) or inhibitor (Mdivi-1 10μm) in combination with TSLP or with TSLP (500ng/mL).*P <0.05, **P < 0.01, ***P < 0.001, **** P<0.0001 using t tests to analyze two independent experiments (nonparametric tests) and one-way ANOVA to analyze in datasets with three or more groups. The graph represents data from at least three replicates. All data are presented as mean ± SD.

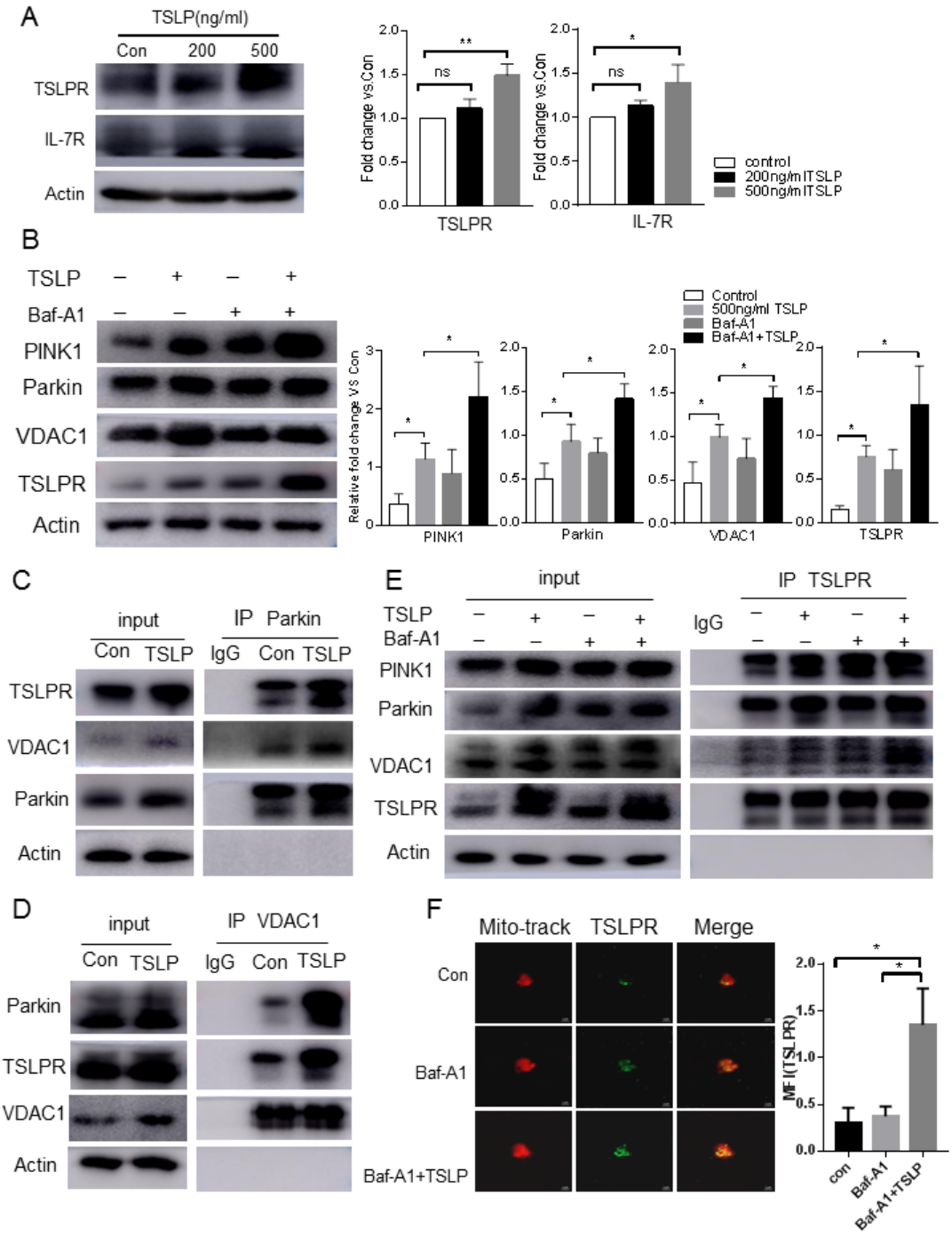

Figure 6

TSLPR binds with Parkin and VDAC1 in platelet mitochondria to regulate mitophagy induced by TSLP. A: Western blot detection and quantification of the expression of TSLP receptors (TSLPR and IL-7R) in platelets treated with TSLP (200ng/mL and 500ng/mL) for 3h. **B:** Human platelets were treated with Baf-A1 (400nM) for 1h, and then TSLP (500ng/mL) for 3h. The expression of mitophagy-associated proteins in platelets was detected and quantified by Western blot. **C-D:** Human platelets were treated with TSLP

(500ng/mL) for 3h, and prepared for immunoprecipitation of Parkin and VDAC1, respectively. **E:** Human platelets were treated with Baf-A1 (400nM) for 1h, then treated with TSLP (500ng/mL) for 3h, and prepared for immunoprecipitation of TSLPR. **F:** Human platelets were treated with Baf-A1 (400nM) for 1h, then treated with TSLP (500ng/mL) for 3h, stained with Mitotracker and TSLPR antibody, then imaged by confocal microscopy. *P <0.05, **P < 0.01 using one-way ANOVA to analyze in datasets with three or more groups. The graph represents data form at least three replicates. All data are presented as mean ± SD.

Supplementary Files

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