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Platelets Exacerbate Cardiovascular Inflammation in a Murine Model of Kawasaki Disease Vasculitis

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Short title: Impact of platelets on murine KD vasculitis

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Kawasaki disease (KD) is the leading cause of acquired heart disease among children. Increased platelet counts and activation are observed during the course of KD, and elevated platelet counts are associated with higher risks of developing intravenous immunoglobulin (IVIG) resistance and coronary artery (CA) aneurysms. However, the role of platelets in KD pathogenesis remains unclear. Here, we analyzed transcriptomics data generated from the whole blood of KD patients and discovered changes in the expression of platelet-related genes during acute KD. In the *Lactobacillus casei* cell wall extract (LCWE) murine model of KD vasculitis, LCWE injection increased platelet counts and the formation of monocyte-platelet aggregates (MPAs), upregulated the concentration of soluble P-selectin, and increased circulating thrombopoietin (TPO) and interleukin (IL)6. Furthermore, platelet counts correlated with the severity of cardiovascular inflammation. Genetic depletion of platelets (*Mpl−/−* mice) or treatment with anti-CD42b antibody significantly reduced LCWE-induced cardiovascular lesions. Furthermore, in the mouse model, platelets promoted vascular inflammation via the formation of MPAs, which likely amplified IL1B production. Altogether, our results indicate that platelet activation exacerbates the development of cardiovascular lesions in a murine model of KD vasculitis. These findings enhance our understanding of KD vasculitis pathogenesis and highlight MPAs, which are known to enhance IL1B production, as a potential therapeutic target for this disorder.

**Keywords:** platelets, monocyte-platelet aggregates, Interleukin-1B, NLRP3, Kawasaki disease, vasculitis, coronary artery, thrombosis, LCWE
Kawasaki disease (KD), an acute febrile disease and systemic vasculitis, is primarily reported in children younger than five years (1, 2). Although the first KD diagnosis was made more than 50 years ago, the KD triggering agent(s) remains unidentified; however, the disease is suspected of having an infectious origin (3). KD is the leading cause of acquired heart disease in children in developed countries. KD predominantly affects small- and medium-sized vessels, particularly coronary arteries (CA), and results in the development of CA aneurysms in up to 25% of untreated affected children (1, 2). CA aneurysms may lead to thrombotic occlusion because of abnormal blood flow conditions in areas of severe dilation (1). Other factors contributing to thrombosis during KD are thrombocytosis, increased platelet activation, adhesion, and endothelial cell dysfunction (1). Furthermore, the ongoing vascular remodeling may lead to coronary stenosis, myocardial ischemia, and even infarction and sudden death (1). The standard of care for KD patients is a single dose of intravenous immunoglobulin (IVIG) and high-to-moderate dose aspirin in the first 10 days of disease onset. This treatment reduces inflammation and decreases the incidence of CA aneurysms to 3-5% (1). However, up to 20% of KD patients are IVIG-resistant and at higher risk of developing CA aneurysms, indicating a need for additional therapeutic approaches (1, 2, 4).

Acute KD patients also exhibit elevated levels of circulating Interleukin (IL)1B, which are reduced following IVIG treatment (5, 6). Indeed, the analysis of whole blood of KD patients indicates increased gene expression related to the NLRP3-IL1B pathway (7). IL1B is a pro-inflammatory cytokine crucial for host defense against infections; however, dysregulated IL1B production is associated with the pathogenesis of several inflammatory disorders (8). Among immune cells, macrophages and monocytes are a major source of IL1B (8, 9). While IVIG is the standard of care for KD patients, multiple case reports and a phase II open-label study demonstrated the safety and efficacy of blocking the IL1 pathway using Anakinra, an IL1 receptor antagonist, to treat KD patients refractory to IVIG treatment (10-13).

Platelets and platelet-mediated release of granules, microparticles, and mediators from activated platelets can influence vascular function and may contribute to the remodeling of the
vasculature (14-17). Such remodeling may include narrowing of the vessel wall or destruction of proteins in extracellular matrix such as collagen and elastin, and remodeling the vascular intimal endothelial cells (14-17). In addition, growing evidence indicates that platelets, critical mediators of hemostasis and thrombosis (18), also interact with innate immune cells (19), and endothelial cells, to amplify inflammation and impact the pathogenesis of several vascular inflammatory disorders, including atherosclerosis and myocardial infarction (20-23). Platelets can boost NLRP3 inflammasome activation and IL1B production in human monocytes through a non-cell-to-cell-contact dependent mechanism via the release of soluble mediators (19). Activated platelets can also aggregate with leukocytes (leukocyte-platelet aggregates), boosting leukocytes' pro-inflammatory functions (21, 24, 25). In particular, activated platelets upregulate P-selectin (CD62P) at their surface, which binds with P-selectin glycoprotein ligand 1 (PSGL1) on monocytes, resulting in the formation of monocyte-platelet aggregates (MPAs) (26-28). Indeed, MPA accumulation in the circulation has been proposed to represent a robust biomarker of platelet activation (22, 23). By forming MPAs, platelets enhance monocyte inflammatory functions (19, 29), and blood MPAs are increased in cardiovascular and inflammatory diseases (23, 30). Platelets release mediators that boost the expression and secretion of pro-inflammatory cytokines by human monocytes, such as IL1B, which are highly inflammatory and important mediators of cardiovascular diseases (31), including KD vasculitis (32).

Increased platelet count, or thrombocytosis, is a common feature of KD patients and is typically reported in the 2nd to 3rd week after disease onset, usually when CA aneurysms appear (1, 33, 34). In addition, higher peak platelet count has been observed in patients with CA aneurysms and IVIG resistance (35, 36). KD patients exhibit enhanced platelet aggregation and increased expression of platelet activation markers, such as platelet-derived microparticles, vascular endothelial growth factor (VEGF), platelet factor 4 (PF4), and β-thromboglobulin (37-43). The frequencies of MPAs and neutrophil-platelet aggregates are increased in KD patients during the acute phase of the disease (43, 44). However, if and how
platelets contribute to the pathogenesis and cardiovascular lesions associated with KD remains unclear.

The *Lactobacillus casei* cell wall extract (LCWE) murine model of KD vasculitis closely mimics the important histopathological, functional, and immune features of human KD vasculitis (32). As observed in KD patients, the LCWE model is also dependent on the NLRP3-IL1B axis, and blocking the IL1 pathway genetically or using Anakinra reduces the severity of the disease (7, 45, 46). Previous studies indicate that in LCWE-induced KD vasculitis, monocytes and macrophages are the main sources of IL1B (46, 47), while neutrophils and eosinophils also express transcripts (*Il1b, Nlrp3, Casp1*, and *Pycard*) and have the capacity to produce IL1B (7). Analysis of autopsied heart tissues from KD patients and cardiovascular lesions of LCWE-injected mice indicates infiltrations of neutrophils, monocytes, and macrophages into the inflamed CA, which can produce IL1B (7, 46-49). Here, we investigated the role of platelets in LCWE-induced KD vasculitis and found increased platelet counts in the first 2 weeks, which correlated with the severity of the cardiovascular lesions. Mice with LCWE-induced KD vasculitis showed increased frequencies of MPAs associated with elevated levels of circulating IL1B. Depleting platelets in mice either by using anti-CD42b antibody or using thrombocytopenic *Mpl−/−* mice resulted in decreased severity of LCWE-induced KD vasculitis and cardiovascular lesions. Of interest, analysis of human transcriptomics data generated from the whole blood of KD patients also showed increased expression of genes associated with platelets and their activation. Thus, our studies suggest that platelets contribute to the development of cardiovascular lesions in the LCWE model, potentially by upregulating IL1B production via MPAs formation. This work provides a rationale for anti-platelet therapy and targeting MPAs to inhibit the development and progression of cardiovascular lesions associated with KD vasculitis.
RESULTS

*Increased expression of platelet-related genes in KD patients.*

To assess the contribution of platelets to KD vasculitis, we used publicly available gene expression datasets generated from KD patients to analyze the expression of a platelet gene signature comprising 40 genes either expressed by platelets or associated with platelet activity ([Figure 1A](#)) (19, 50, 51). Using two different transcriptomic datasets generated from whole blood of acute KD patients and healthy controls (GSE68004 and GSE73461) (52, 53), we observed that 25 and 29 genes from the platelet gene signature were differentially expressed (adjusted *p*-value < 0.05). Among these differentially expressed genes, 22 and 26 were upregulated in acute KD patients (fold change (FC)>1.5) when compared with controls ([Figure 1B, C](#)). We next identified 19 of the platelet signature genes that were significantly and commonly upregulated during acute KD in these two datasets (GSE68004 and GSE73461) ([Figure 1D](#)). Using gene expression data generated from whole blood of acute KD and IVIG-treated convalescent KD patients (GSE63881)(54), we observed that these 19 genes commonly upregulated during acute KD were differentially expressed (adjusted *p*-value < 0.05) in IVIG-treated convalescent patients ([Figure 1E](#)). The abundance of platelet-specific and associated transcripts in pediatric KD vasculitis indicates increased platelet activity and warrants further investigation into their role in KD pathogenesis.

*Increased platelet count during LCWE-induced KD vasculitis.*

To further characterize the contribution of platelets to cardiovascular lesion development during KD, we used the LCWE-induced murine model of KD vasculitis. As previously published (7, 45, 46, 55), compared with PBS-injected control mice, LCWE injection resulted in intense heart vessel inflammation characterized by the development of aortitis and coronary arteritis ([Figure 2A](#)). LCWE injection also led to the development of infrarenal abdominal aorta aneurysms and dilations ([Figure 2B](#)). A time-course analysis indicated that LCWE-injected mice had increased platelet counts starting one week-post LCWE injection and peaking at day 14 post-LCWE ([Figure 2C, D](#)), similar to previous findings described in
KD patients, who show thrombocytosis in the 2nd and 3rd weeks post-illness (34). We next measured the circulating levels of IL6 and thrombopoietin (TPO), both proteins involved in inflammatory thrombocytosis (56, 57). In KD patients, IL6 and TPO levels increase during the first week of illness before the platelet count peaks (58). Similarly, compared with PBS-injected control mice, LCWE-injected mice showed significantly increased levels of circulating levels of IL6 and TPO at one day and one-week post-LCWE, respectively (Figure 2E, F). Finally, in LCWE-injected mice, platelet counts positively and significantly correlated with the development of heart inflammation and abdominal aorta aneurysms (Figure 2G, H). Taken together, these results indicate that LCWE-induced KD vasculitis and cardiovascular lesion development are associated with elevated platelet counts.

**Platelet depletion decreases the severity of LCWE-induced cardiovascular lesions.**

To determine if increased platelet counts promote the development of LCWE-induced KD cardiovascular lesions, we investigated the effects of platelet depletion in LCWE-injected mice. Thrombocytopenia can be induced in mice by intravenous injection of anti-CD42b (GP1ba) antibody (19, 22). WT mice were injected with either anti-CD42b or an IgG isotype control 6 hours before LCWE injection and again on day 3 post-LCWE injection (Supplementary Figure 1A). Treatment of LCWE-injected mice with anti-CD42b resulted in a significant decrease in frequencies and absolute numbers of blood platelets compared with either untreated mice or mice that received the IgG isotype control (Supplementary Figure 1B, C). Remarkably, platelet depletion with anti-CD42b significantly reduced the severity of the cardiovascular inflammation and lesions (Figure 3A, B). The development of LCWE-induced abdominal aorta aneurysms, measured by maximal abdominal aorta diameter and abdominal aorta area, was also decreased in anti-CD42b-treated platelet-depleted mice (Figure 3C-E).

IL1B signaling is required for LCWE-induced KD vasculitis, and blocking the NLRP3-IL1 pathway in mice, either genetically by targeting the Nlrp3, Il1b, or Il1 receptor genes, or pharmacologically using Anakinra, IL1 neutralizing antibodies or NLRP3 small molecules
inhibitors, reduces the development of LCWE-induced cardiovascular lesions (7, 45-47). Monocytes and macrophages are the main sources of IL-1 during LCWE-induced KD vasculitis (7, 46, 47). Since platelets bind monocytes to form MPAs, boost their NLRP3 activation, and license monocytes and macrophages to produce higher levels of IL1B (19), we quantified serum levels of IL1B in LCWE-injected mice treated or not with anti-CD42b. As expected, LCWE injection increased the levels of serum IL1B at days 1 and 7 post-injection, but this was significantly reduced in anti-CD42b-treated thrombocytopenic mice (Figure 3F). Immunofluorescent staining of heart tissue sections with the classical platelet marker CD41 demonstrated elevated platelet numbers in proximity to the inflamed CA in LCWE-injected mice, which was normalized in anti-CD42b treated mice (Figure 3G, H). These results demonstrate that platelets promote the development of LCWE-induced cardiovascular lesions, potentially in an IL1B-dependent mechanism.

**Deletion of c-Mpl inhibits LCWE-induced cardiovascular inflammation.**

We found that TPO, which stimulates thrombocytosis, was elevated in LCWE-injected mice (Figure 2E). TPO signals through the c-Mpl receptor, which is expressed by megakaryocyte progenitor cells, megakaryocytes, and platelets (56, 59). To further investigate the impact of platelets on LCWE-induced KD vasculitis in vivo, we used Mpf−/− mice, which are severely thrombocytopenic (60, 61). As previously reported, Mpf−/− mice developed normally, but when compared with WT mice, showed a significant decrease in blood CD61+ cells, a marker predominantly expressed by platelets and megakaryocytes (Supplementary Figure 2). Compared with WT mice, Mpf−/− mice showed reduced heart vessel inflammation following LCWE injection (Figure 4A, B) and were relatively protected from the development of abdominal aorta dilations and aneurysms (Figure 4C, D). Circulating levels of the alarmin Calprotectin (S100A8/S100A9), which are usually increased in immune-mediated inflammatory diseases, are elevated in LCWE-injected mice and KD patients (62). Indeed, analysis of publicly available gene expression data (GSE141072 (63)) showed that LCWE-injected mice had increased expression of S100a8 and S100a9 in their abdominal aorta
compared to PBS-injected mice (Figure 4E). Since platelets might be a source of calprotectin (64, 65), we next assessed serum levels of calprotectin in WT and Mpl−/− mice either injected with PBS or LCWE. Compared to WT mice, thrombocytopenic Mpl−/− mice showed decreased levels of circulating calprotectin both at baseline and after LCWE injection (Figure 4F). These data indicate that the TPO-mediated increase in platelet count contributes to the development of cardiovascular lesions and that platelets may contribute to calprotectin production in the LCWE-induced murine model of KD vasculitis.

Monocyte-platelet aggregates promote increased IL-1β production during LCWE-induced KD vasculitis.

Since the level of soluble P-selectin, which is released by activated platelets, is elevated in patients with KD during the subacute phase of the disease (66, 67), we next measured the circulating levels of soluble P-selectin in LCWE-injected WT and Mpl−/− mice (Figure 5A). Mpl deficiency resulted in significantly reduced levels of soluble P-selectin at baseline and throughout the course of LCWE-induced vasculitis (Figure 5A). Notably, platelet clumps were visible on peripheral blood smears of LCWE-injected WT mice, starting 24 hours post-injection (Supplementary Figure 3). Platelet-leukocyte aggregates, particularly those containing monocytes and neutrophils, are considered markers of inflammation in several disorders, including cardiovascular diseases (23, 68). Platelet-neutrophil aggregates have been reported in KD patients and shown to increase in frequency in patients with CA aneurysms (43). In addition, KD patients also exhibit increased frequencies of MPAs (44). We defined MPAs in flow cytometry as CD11b+ CD61+ CD115+ and observed that the frequencies of MPAs were decreased in LCWE-injected Mpl−/− deficient mice compared to WT mice (Figure 5B, C). Since activated platelets have the capacity to boost inflammasome activation and IL1B production by monocytes and neutrophils (19), we next measured serum levels of IL1B in PBS and LCWE-injected WT mice and thrombocytopenic Mpl−/− mice. While LCWE injection resulted in increased levels of circulating IL1B in both WT and Mpl−/− mice, the magnitude of this elevation was significantly lower in Mpl−/− mice (Figure 5D). We
acknowledge that even though we did not isolate platelets, it is possible that the method of blood collection for platelet count may potentially activate platelets and MPAs formation. However, the same method was used and performed for all the experimental groups. Overall, these results support the notion that the increased platelet number promotes MPA formation and drives further IL1B release in LCWE-induced KD vasculitis, which may exacerbate the development of cardiovascular lesions.
DISCUSSION

While platelets are known to be involved in the development and progression of many inflammatory and vascular diseases, their contribution to the development of the cardiovascular lesions of KD vasculitis has been unclear. The LCWE-induced murine model of KD vasculitis phenocopies the key immunopathologic features of KD, including coronary arteritis and luminal myofibroblast proliferation (32). This mouse model of KD vasculitis has helped establish the critical role of IL1B in this disease, as blocking the IL1 pathway either with Anakinra or using small molecules inhibitors of NLRP3, or using mice deficient for either Il1a, Il1b, Nlrp3 or Casp1, is the most efficient way to prevent or decrease the severity of LCWE-induced KD vasculitis; more effective than targeting other pro-inflammatory mediators (7, 45-47). Using this model, we reveal increased platelet counts in the second week of LCWE-induced KD development, which correlates with the severity of the cardiovascular lesions. Furthermore, platelet depletion, either by an anti-platelet antibody or using thrombocytopenic Mpl−/− mice, results in decreased severity of LCWE-induced KD cardiovascular lesions. We also observed increased frequencies of MPAs and higher circulating levels of IL1B during LCWE-induced KD vasculitis, which were reduced in thrombocytopenic mice. These data support the hypothesis that platelets may contribute to the development and severity of LCWE-induced cardiovascular lesions via an IL1B-driven mechanism. We acknowledge that one limitation of our study is that we only measured platelet counts and did not investigate their function.

Previous studies have reported enhanced spontaneous platelet aggregation and elevated circulating levels of platelet activation markers during the acute phase of KD (37, 67, 69). Our analysis of publicly available transcriptomics datasets indicates that the expression of genes known to be either platelet-specific or strongly associated with platelet activity increases in children with KD compared to healthy controls and decreases during the convalescent phase of the disease after IVIG therapy. In addition, KD patients with CA aneurysms exhibit higher platelet counts and increased circulating levels of the platelet activation marker β-thromboglobulin, hinting at the potential participation of platelets to the
development of cardiovascular lesions (35, 42). However, the role of platelets in the development, progression, and severity of cardiovascular lesions during KD is not well understood, and the specific mechanisms by which platelets may contribute to KD pathology remain undetermined. KD is associated with systemic inflammation and endothelial dysfunction (1). Activated platelets release mediators, such as matrix metalloproteinases (MMPs), growth factors, lipid mediators, cytokines, microvesicles, reactive oxygen species (ROS), galectins, TGFB, and PF4, which may also regulate vascular remodeling by promoting the narrowing of the vessel wall and destruction of the CA (16, 17). Upon vascular injury or endothelial dysfunction, activated platelets adhere to the injured endothelium, and these platelets-released mediators may, therefore, influence vascular function and contribute to the remodeling and stenosis of the CA also observed in KD patients. Levels of circulating endothelial cells, which detach from vessel walls and indicate vascular injury, are elevated in KD patients compared with healthy controls, and this increase is even more pronounced in KD patients developing CA aneurysms (70, 71). We hypothesize that this vascular injury may lead to platelet activation, which initiates a vicious cycle by amplifying activation of the NLRP3 inflammasome and IL1B production by monocytes and macrophages, and worsening vessel damage (19, 72). Indeed, we report a positive correlation between high platelet counts and the severity of LCWE-induced cardiovascular lesions and inflammation and reduced lesion formation in the setting of platelet depletion.

Our study suggests that inhibiting the formation of MPAs may have a promising therapeutic potential for KD vasculitis, since MPA formation promotes the release of pro-inflammatory cytokines by monocytes. Theoretically, MPA formation could be inhibited by targeting the binding of P-selectin on platelets and PSGL1 on monocytes. Indeed, it has been shown that in vitro, targeting of P-selectin or PSGL1 strongly decreases MPA formation (29, 73). Similarly, MPA formation can also be lowered in vitro by targeting the platelet receptor P2Y12 (73, 74). Currently, high-dose IVIG together with aspirin is the mainstay of therapy in acute KD (1, 2), but the role and the optimal dose of aspirin during the acute stage of the
disease remain controversial. Furthermore, aspirin is inefficient in blocking the formation of platelet-leukocyte aggregates and MPAs, and does not regulate P-selectin levels (23, 75). In cases of severe or complex CA aneurysms with thrombotic complications, other antiplatelet drugs, such as clopidogrel, are recommended by the American Heart Association (1). Since MPAs participate in both thrombosis and inflammation, and their frequencies are increased in KD patients (44), further studies aiming to determine the potential beneficial effect of pharmacologically targeting MPAs and their IL1B production during KD are warranted.

Notably, however, platelets can also exert their pro-inflammatory functions on human macrophages and monocytes via a soluble mediator, in a mechanism that does not involve cell-to-cell contact (19, 29). Activated platelets release α-granules-derived cytokines, chemokines, and growth factors, and in vitro, platelet releasate increases the expression of inflammatory transcripts by human macrophages (73, 76). A recent study further supports the crucial role of platelet cytokine-driven transcription factors in licensing the inflammatory immune response of monocytes (29), as disassembling MPAs using a specific monoclonal antibody against human P-selectin does not affect the capacity of platelets to boost monocyte cytokine responses (29).

Circulating levels of the pro-inflammatory alarmin calprotectin (S100A8/A9 or MRP8/MPR14) are elevated in KD patients during the early acute phase of the disease and decrease after IVIG treatment (77, 78). Furthermore, persistence of high levels of calprotectin after IVIG treatment correlates with the development of CA aneurysms (78), and IVIG-resistant KD patients have increased transcript abundance for S100A8 and S100A9 in whole blood when compared with IVIG-responsive KD patients (79). Purified monocytes from KD patients pre-IVIG treatment exhibit increased mRNA expression of S100A8 and S100A9 (78), indicative of their capacity to produce calprotectin. However, calprotectin can also be released by other cell types, including activated neutrophils and platelets, and elevated calprotectin levels are associated with platelet activation (64, 72). Calprotectin concentrations are positively associated with both platelet aggregation and MPA formation in patients with coronary artery diseases (80, 81). In addition, in peripheral artery diseases, activated platelets...
are enriched in MRP14, which increases their P-selectin expression and the formation of MPAs, and promotes the monocyte inflammatory profile by increasing \textit{IL1B}, \textit{CCL2}, and \textit{TNFA} mRNA levels (82). We previously reported that LCWE-injected mice have increased circulating levels of calprotectin (62), and here, we report decreased calprotectin levels in \textit{Mpl}^-^-^-^-^- mice. However, whether platelets are the main source of calprotectin or enhance its release from myeloid cells or other cellular sources during KD remains to be determined. Thus, calprotectin may represent another potential therapeutic target to modulate cardiovascular lesion formation in KD.

Taken together, our data indicate that platelets may participate in the inflammatory immune response during KD pathogenesis, including ongoing vascular inflammation and remodeling after the initial acute stage, and in the development and progression of cardiovascular lesions by increasing MPA formation and the release of IL1B. Our results demonstrate the immune-effector role of platelets in KD and indicate that strategies targeting MPA formation might be beneficial for KD patients in addition to blocking the IL1B pathway. Future studies should be directed at better understanding the role of platelet-leukocyte aggregates in KD vasculitis, their potential use as a prognostic tool, and the therapeutic consequences of inhibiting their formation. These observations emphasize the role of IL1B in the overall pathogenesis of KD vasculitis, providing additional mechanistic insight into elevated IL1B production during KD and highlighting the need for phase III clinical trials with anti-IL1B therapeutic agents, such as Anakinra.
**METHODS**

**Animals.** Wild-type (WT) C57BL/6J and Mpl\(^{-/-}\) (C57BL/6J-Mpl\(^{-/-}\)hlb219/J) mice were purchased from the Jackson Laboratory. Experimental knockout animals were obtained from homozygous breeding, and age- and sex-matched WT mice from our internal colony at Cedars-Sinai were used as controls. Only male mice were used in this study as LCWE injection induces stronger and more consistent cardiovascular lesions in male than female mice (46, 63). Mice were maintained under specific pathogen-free conditions and used according to the guidelines of the institutional animal care and use committee (IACUC) of Cedars Sinai Medical Center.

**LCWE-induced murine model of KD vasculitis.** *Lactobacillus casei* (ATCC 11578) cell wall extract (LCWE) was prepared as previously described (45). Five-week-old male mice were injected intraperitoneally (i.p.) with 500µg of LCWE or an equal volume of PBS and euthanized one to two weeks post-injection as indicated in the figure legends. On the day of euthanasia, blood was collected via retro-orbital bleeding and centrifuged to obtain serum. Mice were then perfused with PBS and heparin through the heart left ventricle, and heart tissues were harvested and embedded in Tissue-Tek® Optimum Cutting Temperature (O.C.T.) compound (Sakura Finetek, Cat. # 4583). Abdominal aortas were dissected, and photographed before embedding in Tissue-Tek O.C.T. The maximal abdominal aorta diameter was determined by measuring with Image J (NIH) 5 different areas separated by 2mm of the abdominal aorta infra-renal portion (below the left renal artery). The infra-renal abdominal aorta area was also measured in Image J. Serial cryosections (7µm) of heart tissues were stained with hematoxylin and eosin (H&E, Millipore Sigma, Cat. # MHS32). Heart tissues histopathological examination and assessment of the severity of cardiovascular lesions (coronary arteries, aortic root vasculitis, and myocarditis) were performed on H&E stained tissue sections by an expert pathologist blinded to the experimental groups, as previously described (45). Briefly, acute inflammation, chronic inflammation as well as connective tissue proliferation were each assessed using the following scoring system: 0 = no inflammation, 1 = rare inflammatory cells,
2 = scattered inflammatory cells, 3 = diffuse infiltrate of inflammatory cells, and 4 = dense clusters of inflammatory cells. Fibrosis was determined using the following scoring system: 0 = no medial fibrosis, 1 = medial fibrosis involving less than 10% of the CA circumference, 2 = medial fibrosis involving 11 to 50% of the CA circumference, 3 = medial fibrosis involving 51 to 75% of the CA circumference and 4 = medial fibrosis involving more than 75% of the CA circumference. All 4 scores were combined to generate a severity score called “Heart inflammation score”, as previously published (45).

**In vivo platelet depletion.** In vivo platelet depletion was performed using an anti-CD42b (GP1ba) antibody (Emfret Analytics, Cat. # R300) or an isotype control IgG (Rat IgG; Emfret Analytics, Cat. # C301). Antibodies were injected intravenously (i.v.) at a dose of 3mg/kg body weight starting 6 hours prior to LCWE injection. Three days later, the administration of anti-CD42b or control IgG was repeated i.v. at a dosage of 4mg/kg.

**Absolute quantification of platelet count.** Blood was obtained from mouse tail vein and collected in BD microtainer® blood collection tubes (BD Biosciences, Cat. # 365974). Platelet quantification was performed on 50µl of mouse blood by the Department of Comparative Medicine at Cedars-Sinai Medical Center (Los Angeles, CA) using a Drew Scientific Hemavet analyzer (GMI Inc.). When indicated, platelet quantification was performed by flow cytometry. Collected blood samples were stained with anti-CD61-PE (Clone 2C9.G2; Biolegend, Cat. # 104307) or an IgG isotype control (Clone HTK888, Biolegend, Cat. # 400907). Cell numbers were calculated by flow cytometry with CountBright Counting Beads (Thermo Fischer Scientific, Cat. # C36950) according to the manufacturer’s instructions. Stained samples were analyzed on a Sony SA3800 Spectral Analyzer (Sony Biotechnology) and the data were processed using FlowJo (Tree Star Inc.).

**ELISAs.** Levels of TPO (R&D systems, Cat. # MTP00), IL6 (BD Biosciences, Cat. # 555240), S100A8/S100A9 heterodimer (R&D systems, Cat. #DY8596-05) and P-selectin (R&D systems, Cat. # DY737) were measured in the serum of PBS and LCWE-injected mice by ELISA according to the manufacturer’s protocol. IL1B was quantified in serum using the V-
PLEX Mouse IL1B Assay (Meso Scale Diagnostics, Cat. # K152QPD-1) per the manufacturer's instructions using the MSD QuickPlex SQ120 instrumentation and Workbench 4.0 Software (Meso Scale Diagnostics).

**Immunofluorescence.** Frozen heart tissues were collected from mice injected with either PBS, LCWE or LCWE + anti-CD42b. Serial cryosections (7µm) of heart tissues were generated, fixed in acetone and stained with CD41-PE (Clone MWReg30, Biolegend, Cat. # 133905) or a PE IgG isotype control (Clone RTK2071, Biolegend, Cat. # 400907) antibodies to detect platelets in heart tissue sections. Images were obtained using a Biorevo BZ-9000 (Keyence) fluorescent microscope. The quantification of CD41-positive cells per mm² in the tissues surrounding coronary arteries was done with Image J (NIH).

**Flow cytometric detection of platelet-monocyte aggregates.** Whole blood was collected from PBS or LCWE-injected mice and stained with CD61-PE (Clone 2C9.G2, Biolegend, Cat. # 104307), CD11b-PerCP-Cy5.5 (Clone M1/70, Tonbo Biosciences, Cat. # 65-0112-U100) and CD115-Alexa Fluor 488 (Clone AFS98, eBioscience, Cat. # 53-1152-82). The following antibodies were used as isotype controls: Hamster IgG-PE (Clone HTK888, Biolegend, Cat. # 400907), Rat IgG2b-PerCP-Cy5.5 (Clone Tonbo Biosciences, Cat. # 65-4031-U100) and Rat IgG2a-Alexa Fluor 488 (Clone eBR2a, eBioscience, Cat. # 53-4321-80). Stained cells were analyzed on BD FACSaria III (BD Biosciences) and the data were processed using FlowJo (Tree Star Inc.).

**Whole blood smear.** 24 hours after PBS or LCWE injection, a small drop of blood was placed on a glass slide and spread. The smear was allowed to dry and fixed in methanol followed by staining in Wright-Giemsa stain solutions Camco Quik Stain II (Thomas Scientific, Cat. # C872R02) according to manufacturer’s instructions. Images were obtained with the Biorevo BZ-9000 (Keyence) fluorescent microscope.

**Analysis of gene expression datasets.** The publicly available gene expression datasets GSE68004 (52), GSE73461 (53) and GSE63881(54) were obtained from NCBI Gene
expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/). Transcriptomic data analysis was run by GEO2R software as part of the GEO database, and summary statistics were generated with the limma topTable function. The following list of genes was selected as a "platelet gene signature" as previously published (19): C6orf25, CA2, CABP5, CXCL1, CXCL5, F13A1, F2RL1, GNG11, GP1BA, GP1BB, GP9, GRAP2, HIST1H2BK, HIST1H3H, ITGA2B, ITGB3, MYL9, NRGN, PDGFA, PDLIM1, PDZK1IP1, PF4, PF4V1, PLA2G7, PPBP, PRKAR2B, PTCRA, PTGS1, RSG18, SDPR, SELP, SMOX, SPARC, TBXA2R, TGFBI1, THBS1, TMSB4X, TUBB1, VEGFA, ZNF185. This list of platelet signature genes was selected from direct comparisons of publicly available transcriptomes of purified human and mouse platelets (51, 83, 84) and reflects genes that are exclusively or abundantly expressed by platelets.

Expression of the platelet gene signature was first assessed in whole blood of acute KD patients and healthy control (HC) patients (n=37 HC and n=76 acute KD patients for GSE68004 (52); n=55 HC and n=77 acute KD patients for GSE73461 (53)). Differentially expressed genes (DEGs) (Benjamini-Hochberg adjusted p-value <0.05 and fold change (FC)>1.5) from the platelet gene signature were identified in these two datasets. DEGs in both datasets (n=19; CXCL1, F13A1, F2RL1, GNG11, GP9, HIST1H2BK, HIST1H3H, ITGA2B, ITGB3, MYL9, NRGN, PPBP, PTCRA, PTGS1, SDPR, SMOX, SPARC, TUBB1, ZNF185) were then selected, and their expression subsequently analyzed in another dataset generated from whole blood of acute KD patients (n=146) and IVIG-treated convalescent KD patients (n=145) based on a Benjamini-Hochberg adjusted p-value <0.05 (GSE63881) (54). The expression of S100a8 and S100a9 transcripts was determined in a murine RNA-Seq data set generated from abdominal aortas of PBS-injected (n = 5) and LCWE-injected (n = 5) mice (GSE141072) (63). Normalization and analysis of gene expression data were performed in R using edgeR and limma-voom, as previously published (63). S100a8 and S100a9 genes were considered DEGs with an adjusted $P < 0.05$ and FC $\geq 1.5$. Heatmaps showing the expression relative to the mean of selected genes were generated in R with the “ComplexHeatmap” package.
Statistical analyses. Statistical analysis was performed using Prism software (GraphPad). Data normality was determined using the Shapiro-Wilk normality test with alpha=0.05. For two group comparisons, Student’s unpaired two-tailed t-test was used for normally distributed data, and Mann-Whitney two-tailed test was used for non-normally distributed data or for groups with n less than 7. For more than 2 groups, 1-way ANOVA with Tukey post-test analysis was used for normally distributed data. For multiple comparison testing, significance was evaluated by 2-Way ANOVA with Bonferroni’s post-test. The correlation was calculated with the Pearson correlation coefficient. Results are reported as means ± SEM, where each point represents one sample. A p-value of p<0.05 was considered statistically significant. All schematics and the graphical abstract were done with Biorender.

Study approval. All animal studies in this manuscript were approved by the IACUC of Cedars Sinai Medical Center and were performed in accordance with the Guide for the Care and Use of Laboratory Animals.

Data availability. Data supporting the findings of this study are available from the corresponding author upon reasonable request. The publicly available gene expression datasets GSE68004 (52), GSE73461 (53) and GSE63881(54) were obtained from NCBI Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/). The murine RNA-Seq data set generated from the abdominal aortas of PBS- and LCWE-injected mice (GSE141072) (63) was downloaded from NCBI Gene expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/). The platelet gene signature was selected from direct comparisons of publicly available transcriptomes of purified human and mouse platelets (51, 83, 84) and reflects genes that are exclusively or abundantly expressed by platelets. The remaining data are available within the article or supplemental information.

AUTHOR CONTRIBUTIONS
BK, NN, MA, MNR, and M Arditi conceptualized the study. BK, YL, NN, MA, DM, ML, DB, SC, and RP performed experiments. MNR and M Arditi supervised experiments. Data analysis was performed by BK, YL, NN, MA, and MCF. Data discussion was contributed by BK, NN,
MA, SC, RP, MNR, and M Arditi. Manuscript writing was contributed by BK, YL, MNR and M Arditi. The order of equally contributing authors was decided by contribution, seniority and funding of the study.

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FIGURE LEGENDS

Figure 1. Change in the expression of platelet signature genes during acute KD.

(A) List of the 40 genes either expressed by platelets or associated with platelet activity included in the platelet signature genes. (B, C) Heatmaps showing platelet signature genes that are differentially expressed (at least 1.5-fold change (FC) in either direction with an adjusted p-value < 0.05) in whole blood from patients with acute KD compared with HC ((B) GSE68004; KD patients n=76 and HC n=37 and (C) GSE73461; KD patients n=77 and HC n=55). (D) List of 19 genes from the platelet signature genes that were commonly upregulated during acute KD in (B, C). (E) Expression of common platelet signature differentially expressed genes (DEGs) (D) in whole blood of acute KD patients compared with convalescent IVIG-treated KD patients (GSE63881; acute KD patients n=146 and convalescent KD patients n=145). Genes selected based on adjusted p-value<0.05. (B, C and E) Blue-red color gradient: low to high expression relative to the mean of each row. Each column represents 1 patient of the defined groups. Differential expression was analyzed with GEO2R. DEGs; differentially expressed genes. HC; healthy control. KD; Kawasaki disease.

Figure 2. Platelet count correlates with the severity of LCWE-induced KD vasculitis.

(A) H&E-stained heart tissue sections and heart vessel inflammation score from PBS- and LCWE-injected mice at 2 weeks after LCWE-injection (n=10 to 14 mice/group). Scale bar: 200 µm. (B) Representative pictures of the abdominal aorta area and maximal abdominal aorta diameter of PBS- and LCWE-injected mice at 2 weeks after LCWE injection (n=10 to 14 mice/group). (C) Four-week time-course analysis of platelet counts in the blood of PBS- and LCWE-injected mice (n=4 to 5 mice/group). (D) Blood platelet count of PBS and LCWE-injected mice at 2 weeks post-injection (n=14 to 24 mice/group). (E, F) TPO and IL6 levels in the serum of PBS- and LCWE-injected mice (n=8 to 12 mice/group) at 24 hours and 1-week post-LCWE injection. (G, H) Correlation of blood platelet counts with heart inflammation score (G) and maximal abdominal aorta diameter (H). Each symbol represents one mouse. Results presented as mean ± s.e.m. *p<0.05, ***p<0.001, ****p<0.0001 obtained by unpaired t-test.
with Welch’s corrections (A, B, D), 2-way ANOVA with Bonferroni’s multiple comparisons tests (C), 1-way ANOVA with Tukey’s multiple comparisons test (E), Kruskal-Wallis with Dunn’s multiple comparisons test (F), and Pearson r correlation test (G, H). CA; coronary artery, Ao; Aorta, TPO; thrombopoietin.

Figure 3. Platelet depletion attenuates the severity of LCWE-induced KD vasculitis. (A, B) Representative H&E-stained heart tissue sections (A) and heart vessel inflammation score (B) from LCWE-injected mice, or LCWE-injected mice that received either the platelet-depleting anti-CD42b antibody or IgG control at 1 week after LCWE-injection (n=7 to 8 mice/group). Scale bars: 200 µm. (C-E) Representative pictures of the abdominal aorta area (C), maximal abdominal aorta diameter (D) and abdominal aorta area measurements (E) from LCWE-injected mice, or LCWE-injected mice that received either the platelet-depleting anti-CD42b or IgG control at 1 week after LCWE-injection (n=7 to 8 mice/group). (F) Levels of IL1B in the serum of PBS- or LCWE-injected mice that received either IgG isotype control or the platelet-depleting anti-CD42b antibody at day 1 or day 7 post-LCWE injection (n=4 to 7 mice/group). (G) Representative H&E staining and immunostaining staining of CD41 (red) in serial heart sections of WT mice injected with PBS, LCWE, or LCWE-injected mice treated with the platelet-depleting anti-CD42b. (H) CD41+ cell counts in the coronary artery of WT mice injected with PBS, LCWE, or LCWE-injected mice treated with the platelet-depleting anti-CD42b. Scale bars H&E: 200 µm, Scale bars IF: 100 µm. Each symbol represents one mouse. Results presented as mean ± s.e.m. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 obtained by 1-way ANOVA with Tukey’s multiple comparisons test (B, D, E, F, G).

Figure 4. Decreased development of LCWE-induced cardiovascular lesions in thrombocytopenic Mpl−/− mice. (A, B) Representative H&E-stained heart tissue sections (A) and heart inflammation score (B) of LCWE-injected WT and Mpl−/− mice 1 week after LCWE injection (n=12/group). Scale bars: 200 µm. (C, D) Representative pictures of the abdominal aorta area (C), maximal abdominal aorta diameter and abdominal aorta area measurements from WT and Mpl−/− mice (n=15-17/group) at 1-week post-LCWE injection. (E) Heatmap
illustrating the expression of S100a8 and S100a9 (at least 1.5 FC with an adjusted p-value < 0.05) in abdominal aorta tissues of PBS- and LCWE-injected mice (n = 5/groups; GSE141072). Blue-red color gradient: low to high expression relative to the mean of each column. Each row represents 1 mouse of the defined groups. (F) Calprotectin levels in the serum of PBS and LCWE-injected WT and Mpl⁻/⁻ mice, at one-week post-injection (n= 5 to 12/group). Each symbol represents one mouse. Results presented as mean ± s.e.m. pool from 2-3 independent experiments. *p<0.05 and ****p<0.0001 obtained by Unpaired t-test with Welch’s correction (B), Unpaired t-test (D), and 2-way ANOVA with Tukey’s multiple comparisons test (F).

**Figure 5. Increased frequencies of circulating platelet-monocyte aggregates (MPAs) during LCWE-induced KD vasculitis.** (A) Serum levels of soluble P-selectin in PBS- or LCWE-injected WT and Mpl⁻/⁻ mice (n=6-7/group). (B, C) Representative flow cytometry plots (B) and frequencies (C) of CD61⁺ CD11b⁺ CD115⁺ MPAs in whole blood of WT and Mpl⁻/⁻ mice at 3 days LCWE injection (n=5-7/group). (D) Serum IL1B concentration in PBS-injected and LCWE-injected WT and Mpl⁻/⁻ mice at baseline and 3 days post-injection (n=6-7/group). Each symbol represents one mouse. Results presented as mean ± s.e.m. **p<0.01, ***p<0.001, ****p<0.0001 obtained by 2-way ANOVA with Bonferroni’s multiple comparisons tests (A, D) and Mann Whitney test (C).
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