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Autonomous role of Wiskott-Aldrich syndrome platelet deficiency in inducing autoimmunity and inflammation

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Abstract

Background: Wiskott-Aldrich syndrome (WAS) is an X-linked immunodeficiency characterized by eczema, infections, and susceptibility to autoimmunity and malignancies. Thrombocytopenia is a constant finding, but its pathogenesis remains elusive.

Objective: To dissect the basis of the WAS platelet defect, we used a novel conditional mouse model (CoWas) lacking Wiskott-Aldrich syndrome protein (WASp) only in the megakaryocytic

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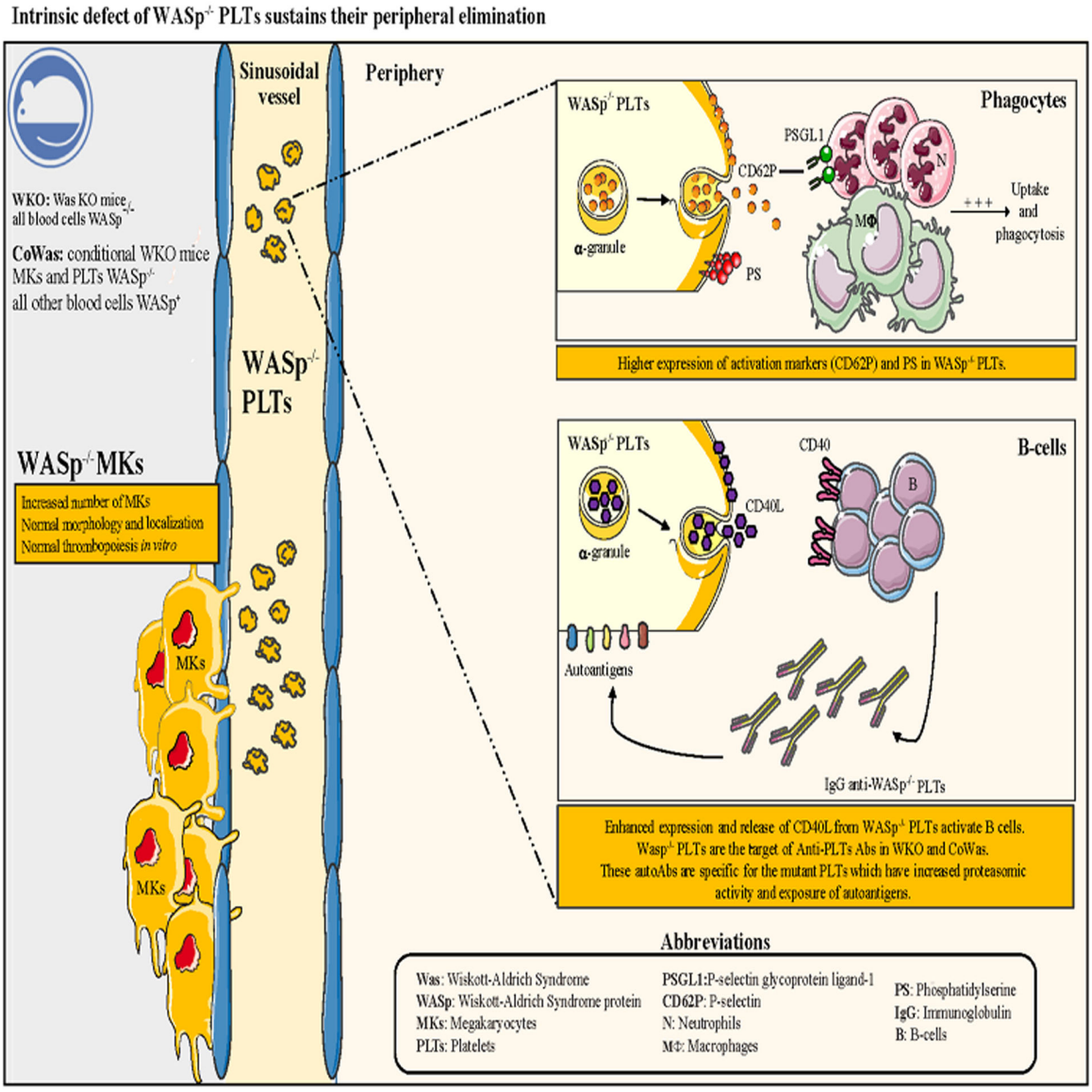
lineage in the presence of a normal immunologic environment, and in parallel we analyzed samples obtained from patients with WAS.

Methods: Phenotypic and functional characterization of megakaryocytes and platelets in mutant CoWas mice and patients with WAS with and without autoantibodies was performed. Platelet antigen expression was examined through a protein expression profile and cluster proteomic interaction network. Platelet immunogenicity was tested by using ELISAs and B-cell and platelet cocultures.

Results: CoWas mice showed increased megakaryocyte numbers and normal thrombopoiesis *in vitro*, but WASp-deficient platelets had short lifespan and high expression of activation markers. Proteomic analysis identified signatures compatible with defects in cytoskeletal reorganization and metabolism yet surprisingly increased antigen-processing capabilities. In addition, WASp-deficient platelets expressed high levels of surface and soluble CD40 ligand and were capable of inducing B-cell activation *in vitro*. WASp-deficient platelets were highly immunostimulatory in mice and triggered the generation of antibodies specific for WASp-deficient platelets, even in the context of a normal immune system. Patients with WAS also showed platelet hyperactivation and increased plasma soluble CD40 ligand levels correlating with the presence of autoantibodies.

Conclusion: Overall, these findings suggest that intrinsic defects in WASp-deficient platelets decrease their lifespan and dysregulate immune responses, corroborating the role of platelets as modulators of inflammation and immunity. (J Allergy Clin Immunol.)

GRAPHICAL ABSTRACT



Keywords

Wiskott-Aldrich syndrome; platelet deficiency; CD40 ligand; autoantibodies; autoimmunity

Wiskott-Aldrich syndrome (WAS) is a severe X-linked immunodeficiency caused by mutations in the WAS gene encoding the Wiskott-Aldrich syndrome protein (WASp), which is specifically expressed in hematopoietic cells and involved in actin polymerization.[1] Patients with WAS have low platelet numbers with reduced size, immunodeficiency, eczema, and high susceptibility to development of tumors and autoimmune manifestations.[2,3] Thrombocytopenia in the absence of other clinical manifestations is referred to as X-linked thrombocytopenia (XLT).[4–6]

Although bleeding episodes occur in the majority of patients and can cause death in 4% to 10% of these patients,[7–9] the pathogenesis of platelet defects remains elusive.[10] Abnormal platelet shedding from megakaryocytes (MKs) and consequent premature bone marrow (BM) release in the presence of normal or increased megakaryopoiesis has been described as a cause of ineffective platelet production.[2,11,12] In contrast, normal megakaryopoiesis and thrombopoiesis associated with increased peripheral elimination were reported by other authors.[13–15] Several studies on platelets performed in *Was*$^{-/-}$ mouse models (WKO) led to contrasting results. The 2 mouse models of WAS[16,17] both show reduced platelet counts but normal mean platelet volume in the absence of bleeding episodes. Studies carried out in these mutants demonstrated defective and premature thrombopoiesis[18] and increased peripheral elimination of *Was*$^{-/-}$ platelets mediated by macrophages and anti-platelet autoantibodies (anti-PLT autoAbs).[19–21] However, because all previous studies were performed in murine mutants or in patients lacking WASp in all hematopoietic lineages, it remains difficult to discriminate the contribution of the defective immune system to platelet defect. To this end, we generated a conditional *Was*$^{-/-}$ mouse model (CoWas) lacking WASp only in the megakaryocytic lineage. Studies performed in CoWas mice allowed us to identify novel intrinsic defects of *Was*$^{-/-}$ platelets, which were in part confirmed also in patients with WAS/XLT. In conclusion, studies in CoWas mice revealed platelet-intrinsic defects causing their increased peripheral elimination independently from other immune system abnormalities and contributing to abnormal modulation of immune responses.

METHODS

Mice

C57BL/6J *Was*$^{-/-}$ mice (WKO) were kindly provided by K. A. Siminovitch[17]; wild-type (WT) mice were purchased from Charles River Laboratories (Calco, Italy); *Rag1*$^{-/-}$ *Was*$^{-/-}$ mice were generated by breeding *Rag1*$^{-/-}$ mice (Jackson Laboratory, Bar Harbor, Me) with WKO mice. CoWas mice were generated by breeding *Was-floxed* female mice[22] and Pf4-Cre male mice (Jackson Laboratory). All the mouse models (WT, WKO, WKO-*Rag1*$^{-/-}$, and *Rag1*$^{-/-}$) were on the C57BL/6J background. Mice were genotyped, as described in the Methods section in this article's Online Repository at www.jacionline.org.

Flow cytometry of murine and human samples

Cell suspensions were stained in fluorescence-activated cell sorting (FACS) buffer (PBS, 0.3% BSA, and 0.1% NaN_3) with antibodies specific for the following markers: anti-CD3 (145–2C11), anti-CD11b (M1/70), anti-B220 (RA3–6B2), anti-Sca1 (D7), anti-CD62P (RB40.34), anti-CD69 (H1.2F3), anti-CD36 (CRF D-2712), anti-CD47 (miap301), and anti-human PAC1 (PAC-1) from BD PharMingen (San Diego, Calif) and anti-CD117 (2B8), anti-CD150 (TC15–12F12.2), anti-CD105 (MJ7/18), anti-CD40L (MR1), anti-CD41 (MWReg30), anti-CD16/32 (93), anti-CD61 (2C9.G2), anti-CD38 (90), anti-CD34 (HM34), Lin$^-$ cocktail, anti-human CD61 (VI-PL2) from BioLegend (San Diego, Calif). Anti-CD42a (Xia.B4) was from Emfret Analytics (Eibelstadt, Germany). Anti-human CD62P (Psel.KO2.3) was from Invitrogen (Carlsbad, Calif). Intracellular WASp staining was performed, as described by Castiello et al.[23] All the flow cytometric samples were acquired

with a FACSCanto II system (BD, Franklin Lakes, NJ) and analyzed with FlowJo software (TreeStar, Ashland, Ore).

Platelet collection and surface staining

Blood collected in Citrate Phosphate Dextrose Solution (Sigma, St Louis, Mo) from the retro-orbital sinus was analyzed with a Sysmex KX-21N hemocytometer (Sysmex, Kobe, Japan). To collect platelet-rich plasma (PRP), blood was then diluted with the same volume of 1× Tyrode buffer (5 mmol/L HEPES, 137 mmol/L NaCl, 2.7 mmol/L KCl, 0.4 mmol/L NaH_2PO_4, and 2.8 mmol/L dextrose, pH 7.4) and centrifuged for 7 minutes at 700 rpm.

For analysis of δ-granules, 5 mL of whole blood was stained with 1 μL of anti-mouse CD61 and incubated for 10 minutes at room temperature. Next, samples were incubated for 30 minutes at 37°C in the dark with 500 μL of mepacrine staining solution (10 μmol/L) in PBS or 500 μL of PBS as negative control.

For reticulated platelet (RT-PLT) staining, 5 μL of whole blood was stained with 1 μL of anti-mouse CD61 and incubated for 10 minutes at room temperature. Next, 1 mL of Thiazole Orange solution (50 ng/mL; Sigma-Aldrich, St Louis, Mo) or PBS as a negative control was added and incubated for 30 minutes at room temperature in the dark. To analyze the staining, we set the gate on WT mice, considering the percentage of RT-PLTs in normal animals as 3% of total platelets.[24]

For Annexin V staining, 200,000 platelets are stained in 100 μL of 1× Binding Buffer with 5 μL of phycoerythrin–Annexin V (BD PharMingen) for 15 minutes at room temperature, followed by stopping reaction by 100 μL of 1× Binding Buffer addition.

To analyze CD40L surface expression, 5 μL of PRP in the presence of 5 μL of agonist was stained with anti-mouse CD61 and anti-mouse CD62P. PRP was incubated with thrombin (final concentration, 1 U/mL; Sigma-Aldrich) for 2 minutes at room temperature. The reaction was stopped by adding 200 μL of FACS buffer and 0.2% paraformaldehyde, and at the end, 1 μL of CD40L antibody per sample is added.

Transmission electron microscopic analysis

Platelet pellets were fixed in 4% paraformaldehyde and 2.5% glutaralde-hyde in 125 mmol/L cacodylate buffer at 4°C for 30 minutes and then in 2% OsO_4 in 125 mmol/L cacodylate buffer for 1 hour. Samples were then washed, dehydrated, and embedded in Epon. Sections were examined on a LEO 912AB transmission electron microscope (Zeiss, Germany; 120 kVand CCD Camera 2048×2048 pixels).

In vivo experiments

Platelet depletion was performed by means of intravenous injection of 0.5 μg/g of rat purified anti-mouse GPIba (Emfret Analytics, Eibelstadt, Germany) in PBS. At different time points after injection, blood has been collected from the retro-orbital sinus to analyze platelet counts by using a hemogram and RT-PLTs by using Thiazole Orange staining. Platelet clearance was evaluated by mean of retro-orbital injection of 3 μg of DyLight 488–labeled or Alexa Fluor 647–labeled anti–GPIb-V-IX (Emfret Analytics) antibody diluted in

PBS.[25] To analyze the percentage of labeled platelets, 10 μL of whole blood was incubated with 2 μL of anti-CD61 for 15 minutes, followed by addition of 150 mL of PBS.

To perform the adoptive transfer experiment, *Was*$^{-/-}$ platelets were isolated form WKO or CoWas mice and stained with the CellTrace Violet Cell Proliferation Kit (Invitrogen) by using 1 μL of the dye every 5 × 10^6 of platelets. Platelets isolated from WT mice were labeled with the CellTrace FarRed Cell Proliferation Kit (Invitrogen) using 1 μL of the dye every 5 × 10^6 platelets. Platelets were mixed in a ratio of 30% WT and 70% *Was*$^{-/-}$ in PBS and injected intravenously into WKO mice (using *Was*$^{-/-}$ platelets isolated form WKO mice) or CoWas recipients (*Was*$^{-/-}$ platelets isolated form CoWas mice). We injected 33 × 10^6 labeled platelets into each recipient. Blood was collected 15 minutes later (time 0) and daily for 6 days; the percentage of Violet$^+$ or FarRed$^+$ platelets was monitored with a FACSCanto II (BD).

To deplete phagocytes, mice were injected intraperitoneally with 100 μL/10 g mouse weight of clodronate liposomes (concentration, 5 mg/mL) at days 0 and 4[26] or PBS liposomes. Blood was collected at day 0 (before injection) and day 7 to evaluate platelet counts.

ELISA

Anti–double-stranded DNA antibodies were evaluated by means of ELISA, as described by Bosticardo et al.[27] The presence of anti-PLT autoAbs was assessed, as described by Brigida et al.[28]

ELISA kits to evaluate concentrations of murine soluble CD62P (sCD62P; eBioscience, San Diego, Calif) and plasma soluble CD40 ligand (sCD40L; eBioscience, San Diego, Calif) were used according to the manufacturer's instructions.

MK analysis

BM cells were stained and MKs were identified as CD41$^+$CD61$^+$ cells by using flow cytometry to analyze the number and frequency of MKs. This analysis was performed in untreated animals 18 hours after treatment with anti-GPIba antibody. MK colony-forming units were analyzed with the MegaCult-C Complete Kit with Cytokines (STEMCELL Technologies, Vancouver, British Columbia, Canada), according to the manufacturer's instructions. Visible MK colony-forming units with purple nuclei and brown granules were counted (4×) in a wide-field microscope. Ploidy distribution and RT-PLT formation have been evaluated as described in the Methods section in this article's Online Repository.

B-cell and platelet culture system

CD43$^+$ cells were depleted from the spleen by using anti-CD43 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) with the AutoMacs Pro Separator (Miltenyi Biotec). One hundred microliters of PRP containing at least 200,000 platelets/mL was incubated for 1 hour at 37°C in a thermomixer (300–350 rpm). Platelet-poor plasma (PPP) was collected on double centrifugation at 8000g for 3 minutes. We plated 150,000 CD43$^-$ cells (resting naive B cells) in the presence of 100 μL of PPP supernatants or complete RPMI as negative controls for 72 hours; CD69 expression was analyzed by using flow cytometry.

Human platelet activation analysis

PRP was collected by centrifuging blood for 10 minutes at 700 rpm. Two hundred thousand platelets per sample were stained for 10 minutes at room temperature in FACS buffer and then fixed with 0.2% paraformaldehyde. The ratio of CD62P/CD61 mean fluorescence intensity (MFI) or PAC1/CD61 MFI[9] was used to normalize the expression level of activation markers for platelet volume, assigning to the healthy donor (HD) unstimulated sample a value of 100.

Sample preparation and proteomic analysis

PRP was centrifuged and lysed through 3 quick freeze/thaw cycles, and then protein extraction was performed by adding RapiGest SF at 0.2% (wt/wt), according to the manufacturer's protocol (Waters, Milford, Mass). Detailed description of proteomic analysis, data processing, and interaction network reconstruction are described in the Methods section in this article's Online Repository.

Statistical analysis

All results are expressed as means and SDs, if not stated otherwise. To assess significance, we used 1-way ANOVA with the Bonferroni postcorrection test or 1-way ANOVA when specified. We also used the 2-tailed Mann-Whitney test, where specified. *P* values of less than .05 were considered significant.

Study approval

Animal procedures were performed according to IACUC 557 and 741 approved by the Italian Institutional Animal Care and OSR Committee. Human studies were performed according to the Helsinki Declaration and the TIGET PERIBLOOD or TIGET08B, TIGET02, and TIGET06 clinical protocols approved by the OSR Ethical Committee. The study was also approved by the National Institutes of Health Institutional Review Board (protocol 16-I-N139).

RESULTS

CoWas mice selectively lack WASp in platelets

To understand the extent to which the lack of WASp expression affects platelet generation and survival, we generated mice lacking WASp only in the megakaryocytic lineage. We obtained compound heterozygous female ($CoWas^{+/-}$) and CoWas mice, which were completely null for WASp expression in the megakaryocytic lineage (see Fig E1, *A*-*C*, in this article's Online Repository at www.jacionline.org). Intracellular WASp expression was assessed by using flow cytometry on WBCs and platelets isolated from the peripheral blood of age-matched mice. As expected, WKO mice lacked WASp expression both in WBCs and in platelets, whereas CoWas mice lacked WASp selectively in platelets. $CoWas^{+/-}$ mice showed a double population of $WASp^{+}$ and $WASp^{-}$ platelets in the presence of normal WASp expression in WBCs (Fig 1, *A*). $CoWas^{+/-}$ female mice showed more than 50% $WASp^{+}$ platelets, suggesting a selective advantage for $WASp^{+}$ platelets in the periphery (Fig 1, *B*). CoWas and WKO mice showed comparably low platelet counts ($446.6 \pm 69.62 \times 10^{6}$

platelets/mL and 495 ± 105.9 × 10^6 platelets/mL, respectively), whereas CoWas$^{+/-}$ mice showed intermediate platelet counts between WT and mutant mice (711.2 ± 89.23 × 10^6 platelets/mL; Fig 1, *C*). No differences in mean platelet volume, WBC counts, or hematocrit values were observed among mutants and control animals (Fig 1, *D*, and data not shown). Analysis of glycoproteins expressed on the platelet surface showed no alterations in mutant mice (see Fig E1, *D*).

To further characterize morphologic changes in *Was*$^{-/-}$ platelets, we analyzed the size and granule content using transmission electron microscopy (TEM; Fig 1, *E*). In contrast to human findings,[2,8,29] platelets from WKO and CoWas mice did not show reduction in size (Fig 1, *F*, left panel), as previously reported in WKO models.[16,18] Consistently, the quantity of α-granules and dense granules (δ-granules), both containing factors responsible for platelet activation and aggregation, was similar in all 3 groups of mutant mice (Fig 1, *F*, right panel).

Thrombopoiesis in the absence of WASp

To test whether peripheral thrombocytopenia could be due to defective thrombopoiesis, we analyzed the megakaryocytic compartment. WKO and CoWas mice showed an increased percentage of MK progenitors (CD150$^+$CD41$^+$ on Lin$^-$c-Kit$^+$Sca1$^-$ cells Fig 2, *A*)[30] and a greater number of megakaryocytic colony-forming units (Fig 2, *B*). Further analysis to better dissect MK development showed an increase in MK progenitors from the stage of promegakaryocytes (CD34$^-$ CD38$^+$CD41$^+$CD61$^+$CD42a$^{+/-}$ cells; see Fig E2, *A*, in this article's Online Repository at www.jacionline.org).[31] Of note, no differences in ploidy distribution of MKs were observed in mutant compared with control animals (see Fig E2, *B*). Analysis of the percentage and absolute number of mature MKs (CD41$^+$CD61$^+$) revealed a significant increase in both CoWas and WKO mice, which is in line with data reported in the literature (Fig 2, *C* and *D*).[18,19] All these findings suggest that peripheral thrombocytopenia stimulates megakaryopoiesis to compensate for the peripheral defect.

To assess the efficiency of thrombopoiesis *in vivo*, we counted RT-PLTs[24] using Thiazole Orange staining. WKO and CoWas mice showed a decreased percentage of RT-PLTs (Fig 2, *E*), which might suggest impaired thrombopoiesis. However, MK analysis in the BM of mutant mice assessed by using immunohistochemistry with anti-CD31 antibody (depicting sinusoids and MKs) and anti-Factor VIII antibody (von Willebrand factor, labeling MKs) did not reveal any mislocalization (data not shown). Also, *Was*$^{-/-}$ MKs showed *in vitro* normal proplatelet formation (see Fig E2, *C-E*).

To test *in vivo* thrombopoiesis, we depleted platelets by means of platelet injection of anti-GPIbα mAb (Fig 2, *F*). Daily blood collection demonstrated platelet recovery to predepletion values within 3 to 4 days in all mice (Fig 2, *G*). Of note, no effect of anti-GPIbα treatment on MK numbers has been found using WT animals (see Fig E2, *F*); however, we cannot exclude an effect of WASp deficiency on the recovery of the functional MK pool after GPIb treatment. Additionally, evaluating the percentage of RT-PLTs after anti-GPIba treatment, we found that RT-PLTs from mutant mice reach an equilibrium with the same kinetics of WT mice (see Fig E2, *G*). Although these *in vivo* data might suggest normal thrombopoiesis in WKO and CoWas mice, at least in stressed situations, because

mutant animals have higher numbers of MKs in the BM and do not reach an equilibrium of RT-PLTs in a shorter time period, we cannot exclude that thrombopoiesis per MK could be affected.

Increased peripheral elimination of hyperactived *Was*$^{-/-}$ platelets in CoWas mice

To test the increased peripheral platelet elimination hypothesis, we assessed platelet half-life by means of *in vivo* injection of Dy-Light 488-anti-GPIb-V-IX antibody selectively labeling circulating platelets[25] and observed a shorter lifespan in WKO and CoWas mice (Fig 3, *A* and *B*), although we cannot exclude an effect of the antibody on platelet clearance. To further characterize this defect, we transferred a mixed population of differently labeled WASp^{+} (from WT) and WASp^{-} (from WKO or CoWas) platelets into WKO and CoWas recipients. Specifically, labeled platelets were mixed at a ratio of 30% WT and 70% CoWas or 30% WT and 70% WKO and infused intravenously into CoWas and WKO mice, respectively. The proportion of WASp^{+} platelets increased over time, whereas proportions of WASp^{-} platelets decreased in all recipients, indicating that WASp^{-} platelet clearance is independent of the host environment (Fig 3, *C*) and suggesting an intrinsic platelet defect.

To test phagocyte contribution in mediating peripheral platelet elimination, we injected clodronate liposomes to deplete macrophages *in vivo*.[26,32] Analysis of the frequency of F4/80^{+} cells in the spleens of clodronate liposome-treated mice confirmed macrophage depletion (data not shown). Seven days after administration, WKO and CoWas mice showed a significant increase in platelet numbers, indicating a critical contribution of phagocytes to peripheral elimination of WASp-deficient platelets (Fig 3, *D*). Platelet increase was more obvious in WKO mice than in CoWas mice. We speculate that the defective migratory ability of *Was*$^{-/-}$ macrophages in WKO mice could lead to increased platelet elimination within tissues, inducing a more substantial increase in platelet counts after phagocyte depletion. Rapid phosphatidylserine exposure occurring concomitantly to activation is one of the main mechanisms mediating platelet phagocytosis. In line with data from patients with WAS,[15] platelets from both mutant strains showed higher frequencies of Annexin V^{+} platelets (Fig 3, *E*). Increased levels of sCD62P in plasma (Fig 3, *F*) and surface expression of CD62P (Fig 3, *G*) were also found in mutant compared with control platelets. In sum, our data indicate that cell-intrinsic mechanisms cause hyperactivation of WASp-deficient platelets, which are in turn rapidly removed from the periphery by phagocytic cells.

Proteomics of *Was*$^{-/-}$ platelets reveals perturbed metabolism and enhanced antigen processing

To investigate the intrinsic mechanisms underpinning the enhanced activation and removal of *Was*$^{-/-}$ platelets, we evaluated their proteomic profile. More than 1400 proteins were identified (see Table E1 in this article's Online Repository at www.jacionline.org), and 686 proteins were shared among mutant and WT platelets (corresponding to >46% of total proteins; Fig 4 and see Fig E3, *A*-*C*, in this article's Online Repository at www.jacionline.org).

To identify differentially expressed proteins, linear discriminant analysis was applied, and 221 proteins presenting *F* ratios of 3.5 or greater and *P* values of .05 or less were extracted

as descriptors (see Table E2 in this article's Online Repository at www.jacionline.org). Hierarchical clustering obtained on processing descriptors showed that CoWas is an intermediate condition between WT and WKO (see Fig E3, *D*). Of note, the majority of extracted descriptors were confirmed by fold change and DAve analyses (see the Proteomics data processing section in the Methods section). In addition, the effects of WASp deletion were evaluated plotting the selected descriptors into a *Mus musculus* protein-protein interaction network by using Cytoscape and STRING db (see Fig E4 in this article's Online Repository at www.jacionline.org). The network analysis (19 clusters) confirmed intermediate levels of expression of distinct biological processes/functional classes in CoWas platelets between WT and WKO platelets. This finding was more evident when focusing on a subnetwork of 8 clusters involving metabolic processes, cytoskeleton, ubiquitins, 14-3-3 proteins, and immunoglobulins (Fig 5, A). Specifically, we did not find any abnormalities in the total amount of actin-related proteins but relative changes in some proteins related to this pathway. Metabolic profile perturbation with a reduction in lipid, glucose, and phosphate metabolism-related proteins was also found. On the other hand, ubiquitin-related proteins were upregulated in line with reduced lifespan and increased antigen processing, which is frequently observed in patients with autoimmune disorders.[33] The proteomic profile also revealed alterations in the immunoglobulin content, with a substantial increase in platelet-associated IgM, IgA, and IgG levels in WKO mice (Fig 5, *A*). Of note, the ''don't eat me'' signal CD47 molecule decreases in CoWas and WKO mice, as confirmed by using flow cytometric analysis (Fig 5, *B*).

The proteomic profile suggests a scenario in which absence of WASp (1) decreases the platelet lifespan through inhibition of several metabolic pathways and enhancement of ubiquitination and proteasomal activity that (2) increases antigen processing, contributing to triggering of autoimmunity. In addition, our data show that WASp-deficient platelets present intrinsic defects that become more severe in WKO mice, likely because of the aggravating role of platelet-extrinsic immune dysfunction.

Was$^{-/-}$ platelets trigger autoimmune responses in CoWas mice

Because of the presence of anti-PLT autoAbs in patients[34] and WKO mice,[20] we hypothesized that WASp-deficient B cells can react against platelets by producing neutralizing antibodies. In line with this, *Was*$^{-/-}$*Rag1*$^{-/-}$ double-knockout mice (WKO-*Rag1*$^{-/-}$), which lack T and B cells, showed platelet counts comparable with those of WT mice, thus supporting a partial involvement of adaptive immunity in the peripheral elimination of *Was*$^{-/-}$ platelets (Fig 6, *A*) and further supporting data observed in the *μMT*$^{-/-}$ *x Was*$^{-/-}$ model.[20]

To test whether production of anti-PLT autoAbs could be related to B-cell dysregulation[23,35–39] or triggered by the intrinsic immunogenicity of *Was*$^{-/-}$ platelets, we measured anti-PLT autoAbs in CoWas mice. To do this, we used an ELISA detecting serum anti-PLT IgG that was more sensitive than the proteomics approach used above. No autoantibodies against WT platelets were detected in WT mice, whereas autoantibodies against *Was*$^{-/-}$ platelets were present in sera of WKO and CoWas mice. Very importantly, CoWas$^{+/-}$ mice had autoantibodies specifically against *Was*$^{-/-}$ platelets but not against WT

platelets (Fig 6, B). Of note, CoWas mice do not show any signs of autoimmunity and the analysis of serum anti-double-stranded DNA autoantibodies did not detect any positivity (Fig 6, *C*).

Because of the presence of autoantibodies in CoWas mice despite the normal B-cell compartment, we hypothesized that *Was*$^{-/-}$ platelets could sustain humoral autoimmunity by producing immunomodulatory factors that trigger B-cell responses. In this context platelets have been demonstrated to mediate inflammatory and immune responses by releasing chemokines and cytokines, including CD40L, which is highly expressed and released on activation. Platelet-derived CD40L can modulate adaptive immune mechanisms influencing B-cell homeostasis by inducing isotype switching.[40–43] Thus we evaluated platelet CD40L surface expression on *in vitro* activation, finding a significant increase in WKO and CoWas mice (Fig 6, D, left). Consistently higher release of sCD40L was retrieved in mutant PPP obtained from unwashed platelets, possibly reflecting higher activation of *Was*$^{-/-}$ B cells, which can consume sCD40L (Fig 6, *D*, right).

Next, we tested whether the greater expression and release of CD40L could directly modulate B-cell activation. We cultured B cells isolated from spleens of WT, WKO, and CoWas mice in the presence of PPP from WT, WKO, or CoWas mice and analyzed B-cell activation. B cells cultured in the presence of supernatants obtained from platelets lacking WASp showed a higher proportion of the activation marker CD69 (Fig 6, *E*). Overall, these findings suggest that, in the absence of WASp, platelets can contribute to sustaining immune dysregulation by expressing and releasing higher levels of CD40L, which can induce B-cell activation, even in a normal immune environment.

Activated platelets and increased sCD40L in patients with WAS/XLT

To corroborate these findings in human subjects, we evaluated platelet activation and analyzed levels of serum sCD40L in a cohort of patients with WAS with various clinical manifestations and the presence or absence of autoantibodies (see Table E3 in this article's Online Repository at www.jacionline.org).[37,44] Human platelet activation was evaluated in adult HDs and patients by analyzing the expression of CD62P and PAC1, the activated form of integrin $\alpha_{IIb}\beta_3$ (GPIIbIIIa).[45] We found that the MFIs of both CD62P and PAC1 were significantly increased in patients (Fig 7, *A* and *B*). To support the immunomodulatory role of platelets, different autoimmune conditions have been associated with increased serum levels of sCD40L, of which platelets are the major source.[46–48] We tested levels of sCD40L in the plasma of 14 patients with WAS who were also at risk of autoimmune complications and in 19 pediatric age-matched HDs. Higher sCD40L concentrations were found in patients with circulating autoantibodies than in patients with no autoantibodies (Fig 7, *C*, and see Table E3).

Overall, in patients with WAS, platelets are hyperactivated, and in turn, this activation can contribute to their peripheral elimination and further sustain inflammation and B-cell activation, predisposing to autoimmune complications.

DISCUSSION

Thrombocytopenia is a common feature present in both patients with XLT and those with WAS.[2–6] Controversial data on platelet defects have been reported, but all these studies were performed in patients and murine models lacking WASp in all hematopoietic cells, thus preventing a clear dissection of the contribution of intrinsic versus extrinsic factors. To overcome this issue, we generated CoWas mice lacking WASp only in the megakaryocytic lineage. Although no gross morphologic changes were detected in platelets from CoWas mice, the lower absolute count in a normal immune environment strongly suggested an intrinsic defect.

One of the more controversial issues in the setting of WAS-related thrombocytopenia is analysis of the thrombopoietic activity of deficient MKs, and indeed, observations in our mutant models do not completely clarify this complex issue. BM analysis in WKO and CoWas mice showed an increased percentage of MK progenitors and MKs, suggesting that peripheral thrombocytopenia triggers megakaryopoiesis. *In vitro* proplatelet production tests showed that *Was*$^{-/-}$ MKs are able to produce proplatelets at a normal rate compared with WT counterparts. Additionally, after *in vivo* platelet depletion, mutant animals treated with anti-GPIb antibody restored platelet counts with kinetics comparable with those in WT mice. To corroborate our findings, it has been demonstrated recently that human proplatelet formation *in vitro* depends on N-WASp, whereas WASp is dispensable.[49]

Despite this evidence, RT-PLT numbers and percentages in peripheral blood were reduced in both mutants to a level similar to those seen in patients with WAS[20,50]; these data contrast with the work published by Prislovsky et al,[19] who showed a normal frequency of RT-PLTs in WKO mice. This discrepancy might be due to the different genetic background and effect of genetic modifiers present in various mouse models.[16,17] The reduced frequencies and counts of RT-PLTs present in WKO and CoWas mice could also suggest a defective thrombopoiesis, although in contrast with our *in vitro* data. However, a reduced half-life of *Was*$^{-/-}$ RT-PLTs compared with WT RT-PLTs based on the *in vivo* data of mature platelets could be hypothesized. Accelerated kinetics of RT-PLT maturation to compensate for the faster mature platelet elimination might be an additional factor. Moreover, we cannot exclude the hypothesis that in the absence of WASp, an important fraction of platelets could be generated as nonreticulated.[19] Finally, because WKO and CoWas mice have increased MK numbers, thrombopoiesis per MK could still be affected. Overall, because the data reported here are obtained in a stress condition (after severe depletion), defective thrombopoiesis at steady state cannot be excluded.

We speculated that despite the normal immune environment, peripheral platelet destruction in CoWas mice could be the result of an intrinsic platelet defect. In line with this hypothesis, a shorter half-life of *Was*$^{-/-}$ platelets was observed in CoWas and WKO mice. Moreover, mutant mice showed increased sCD62P levels in plasma with no increase in sCD62P mRNA expression (data not shown), indicating that the increased protein expression and serum levels might reflect enhanced a-granule release because of the perturbation of actin polymerization inside the cytosol consistently with the role of WASp in regulation of granule release. Thus WASp deficiency causes dysregulation in the expression and release of

CD62P, which in turn might induce increased uptake of activated platelets by phagocytes through CD62P/PSGL1 interactions.[51–55] Platelet activation also results in rapid exposure of phosphatidylserine, a well-known ‘‘eat- me’’ signal. In line with this, *in vivo* elimination of phagocytes increased platelet counts in WKO and CoWas mice. Additionally, B cells can produce anti-PLT autoAbs, which are frequently detected in patients and WKO mice.[20,34] Remarkably, CoWas mice and even CoWas$^{+/-}$ mice, produce autoantibodies selectively against WASp-deficient platelets. The increased expression and release of CD40L can contribute to tolerance breakdown by promoting B-cell activation and inducing immunotype switching.[23,40,56–59] Indeed, the difference between sCD40L levels retrieved in PPP from WKO and CoWas mice might reflect the altered distribution of B-cell subpopulations. In fact, with similar release of sCD40L by *Was*$^{-/-}$ platelets, CoWas mice have no defects in the B-cell compartment and require a lower amount of sCD40L produced by platelets. On the contrary, WKO mice have hyperactivated B cells that produce higher levels of circulating IgM and IgG at steady state, thus suggesting that higher consumption of sCD40L occurs to sustain B-cell activation and class-switch recombination.[22,59–61]

These data were corroborated further in a small cohort of patients with WAS. We observed greater expression of activation markers in patients; our observations are in contrast with data reported by Gerrits et al,[9] who evaluated platelet activation on whole-blood samples, finding no significant difference between patients and HDs.

We performed analyses of platelet activation on platelets isolated from peripheral blood. The different type of samples analyzed could explain this discrepancy because in whole blood activated platelets could bind to leukocytes, thus underestimating the P-selectin expression. Patients with detectable autoantibodies also showed increased plasma levels of sCD40L, which are likely derived from *in vivo* activation of platelets. In turn, sCD40L increase can sustain inflammation and induce B-cell isotype switching. The proteomic profile of *Was*$^{-/-}$ platelets isolated from WKO but not CoWas mice revealed relevant changes in immunoglobulin content (IgA, IgG_{2b}, IgG_3, and IgM), reflecting perturbation of the B-cell compartment.[23,56–59] In parallel, the proteomic analysis demonstrated that both platelet-intrinsic and platelet-extrinsic mechanisms cooperate in the pathogenesis of WAS-related thrombocytopenia. Proteomic alterations shared between WKO and CoWas mice indicate intrinsic platelet defects because of the absence of WASp, whereas proteins differentially expressed between WKO and CoWas mice reflect the direct influence of the immune system. *Was*$^{-/-}$ platelets from both murine models show metabolic dysregulation, with lower expression of proteins involved in lipid metabolism processes, such as those involved in the electron transport chain or in fatty acid β-oxidation, as well as SLC25A24, a mitochondrial calcium-dependent carrier of Mg-ATP/Mg-ADP that protects against oxidative stress.[62,63] Conversely, WKO platelets upregulated stomatin, a lipid raft component of a-granules involved in platelet activation.[64] Glucose metabolism, protein kinases, phosphates, and relative cascade pathways were downregulated in the absence of WASp.

Interestingly, levels of CD47, a molecule that, when absent, leads to mild spontaneous thrombocytopenia,[65] progressively decrease from CoWas to WKO mice, suggesting an additional mechanism contributing to the induction of phagocytosis. Consistent with the frequent bleeding episodes occurring in patients irrespective of clinical severity, integrin β3,

integrin IIb/IIIa, and some 14-3-3 family proteins were downregulated.[66] On the other hand, upregulation of ubiquitin-related proteins was found. All these findings support the intrinsic defect that renders platelets more prone to apoptosis and concomitantly more capable of processing antigens for presentation to the immune system, a phenomenon that can contribute to the development of autoimmunity.[33]

In conclusion, our study provides novel insights into the pathogenesis of WAS, highlighting the intrinsic role of WASp in platelet turnover and peripheral homeostasis. We shed new light on the mechanisms underlying WAS immune dysregulation, showing that in the absence of WASp, platelets secrete a high amount of CD40L, sustaining inflammation and contributing to the altered B-cell function. Overall, our study identifies platelet defects as contributing to both impaired hemostasis and dysregulated immunity, extending the complex scenario of defective cellular processes occurring in patients with WAS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

Anti-PLT autoAb	Anti-platelet autoantibody
FACS	Fluorescence-activated cell sorting
HD	Healthy donor
MFI	Mean fluorescence intensity
MK	Megakaryocyte

PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
RT-PLT	Reticulated platelet
sCD40L	Soluble CD40 ligand
sCD62P	Soluble CD62P
WAS	Wiskott-Aldrich syndrome
WASp	Wiskott-Aldrich syndrome protein
WT	Wild-type
XLT	X-linked thrombocytopenia

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Key messages

- Selective WAS absence is dispensable for MK function and thrombopoiesis.
- Conditional WAS inactivation in platelets affects peripheral survival, leading to phagocytosis and autoantibody elimination through CD40L.
- *Was*$^{-/-}$ platelets have decreased metabolic activity and increased ubiquitination pathways.
- Increased platelet activation and CD40L plasma levels are observed in patients with WAS.

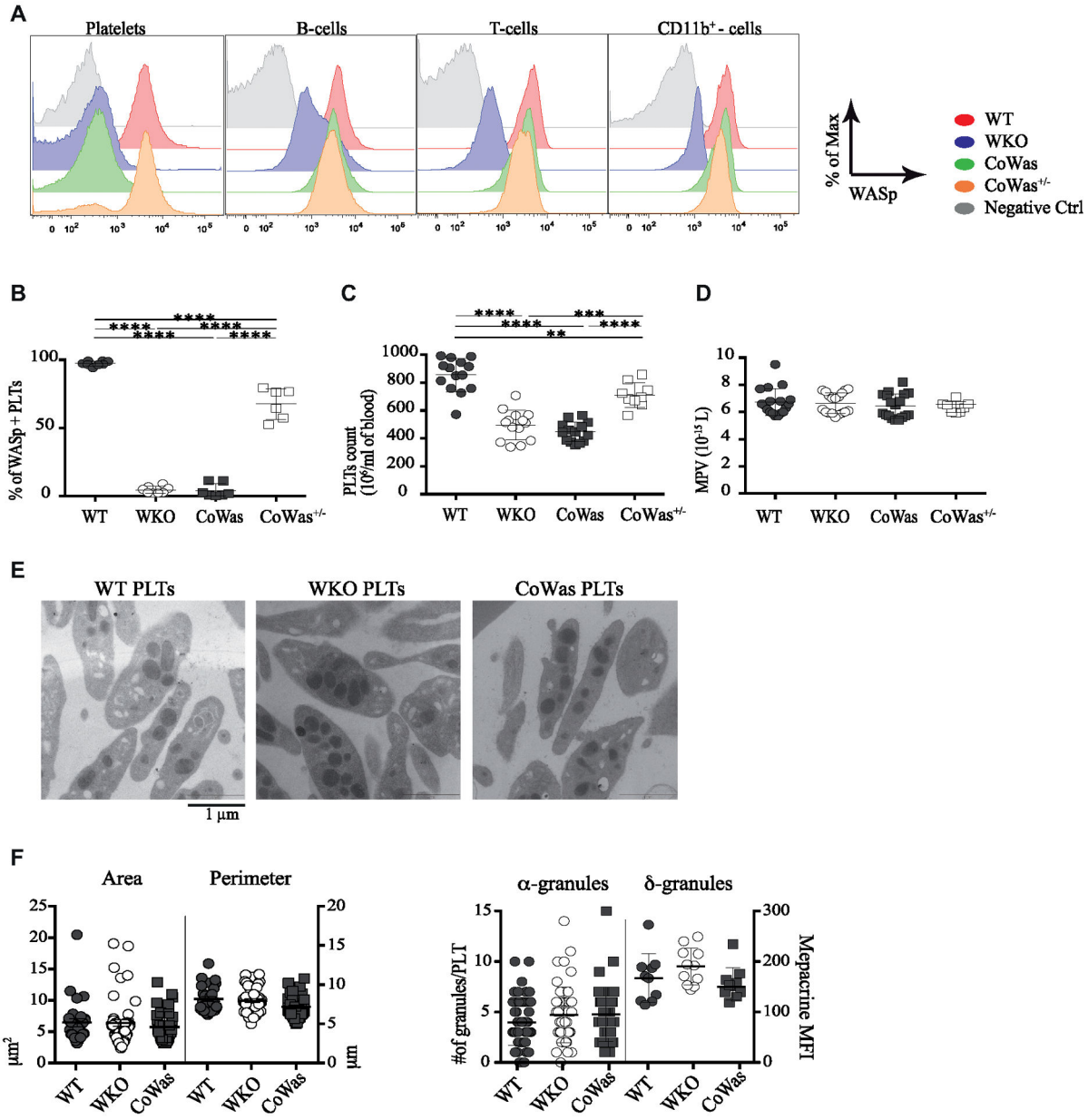

FIG 1.

Characterization of CoWas mice. **A**, WASp expression was evaluated in different cell types in peripheral blood of6-week-old WT, WKO, CoWas, and CoWas$^{+/-}$ mice. **B**, Graph shows the percentage of WASp^{+} platelets in different mouse models at 8 to 12 weeks of age. **C** and **D**, Hemogram analysis of WT, WKO, CoWas, and CoWas$^{+/-}$ mice shows platelet counts (Fig 1, *C*) and mean platelet volume (*MPV*; Fig 1, *D*). **E**, Representative TEM images of platelets isolated from peripheral blood of WT, WKO, and CoWas mice. **F**, The perimeter and area were measured by using ImageJ Software *(left panel)*, as was the number of α-granules *(right panel)*. Dense granules were evaluated by using mepacrine and analyzed by using flow cytometry *(right panel)*. In Fig 1, *B-D*, each *dot* represents a different mouse from 2 independent experiments; in Fig 1, *F*, each *dot* represents the analysis of single

platelets within different TEM images. All the graphs report means ± SDs, and statistical analysis was performed with 1-way ANOVA and the Bonferroni postcorrection test. *$P < .05$, **$P < .005$, ***$P < .001$, and ****$P < .0001$. *PLT*, Platelets.

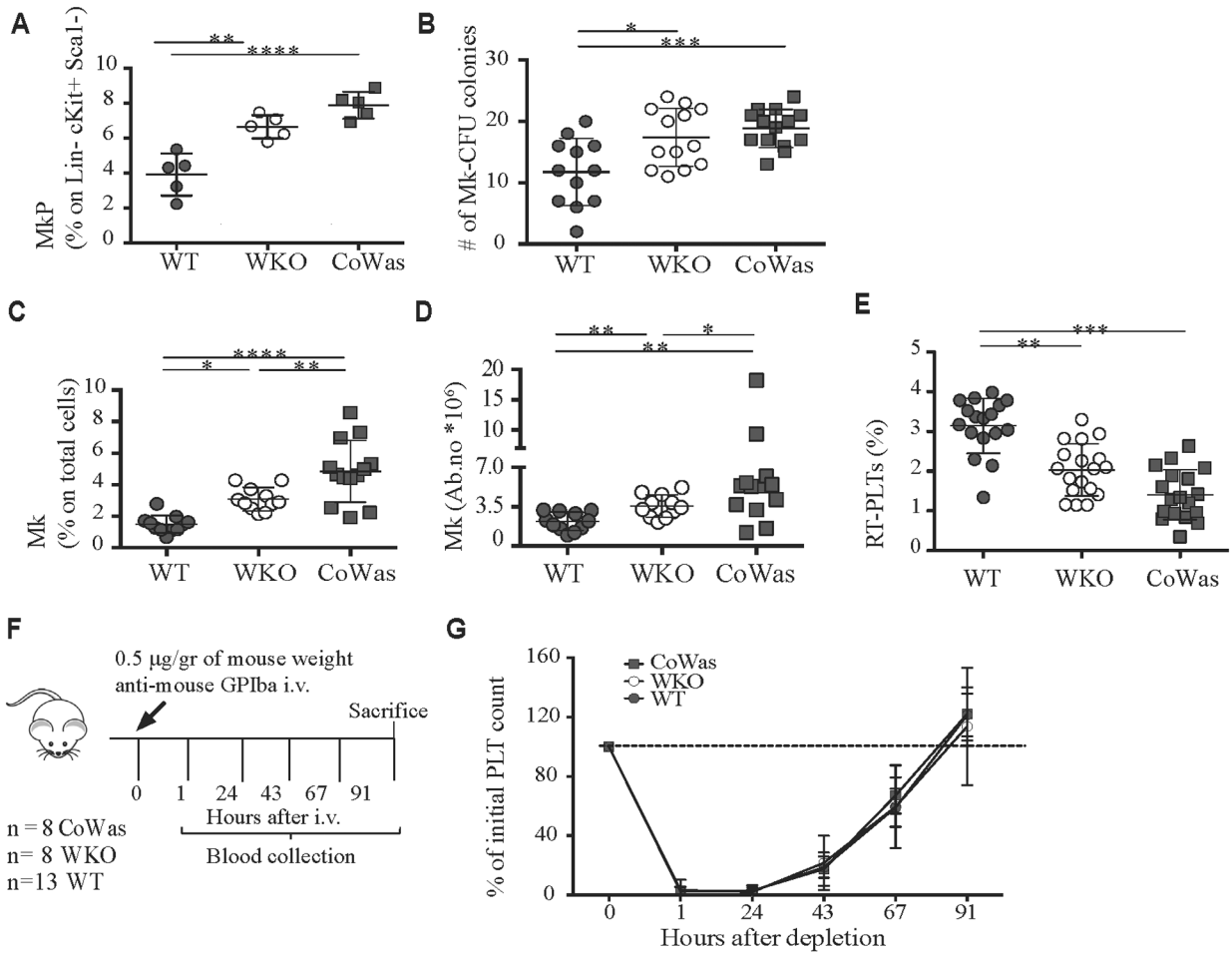

FIG 2.

Phenotypic and functional characterization of the megakaryocytic compartment. **A**, Evaluation of megakaryocyte progenitors *(MkP)* identified as $CD41^{+}CD150^{+}$ cells gated on the $Lin^{-}c\text{-}Kit^{+}Sca1^{-}$ compartment in WT, WKO, and CoWas mice is shown. **B**, The number of MKcolony-forming units was counted with the microscope by using a ×4 objective. **C** and **D**, MKs ($CD61^{+}CD41^{+}$ cells) are expressed as percentages on total BM cells (Fig 2, *C*) or absolute numbers (Fig 2, *D*) and analyzed in the bone marrow of age-matched WT, WKO, and CoWas mice. **E**, The percentage of RT-PLTs is assessed by using Thiazole Orange (TO). **F**, Platelet depletion was performed by using intravenous injection of anti-mouse GPIba (Emfret Analytics) according to the scheme indicated in the figure. **G**, Platelet counts were monitored daily by means of blood collection and expressed as percentages of initial platelet counts at time 0 at different time points. In all graphs each *dot* represents a different mouse from 1 (Fig 2, *A*), 2 (Fig 2, *G*), 4 (Fig 2, *E*), or 6 (Fig 2, *B-D*) independent experiments. All the graphs report means ± SDs, and statistical analysis was performed with 1-way ANOVA (Fig 2, *A-E*) or 2-way ANOVA (Fig 2, *G*) and the Bonferroni postcorrection test. **P* < .05, ***P* < .005, ****P* <.001, and *****P* < .0001. *PLT*, Platelets.

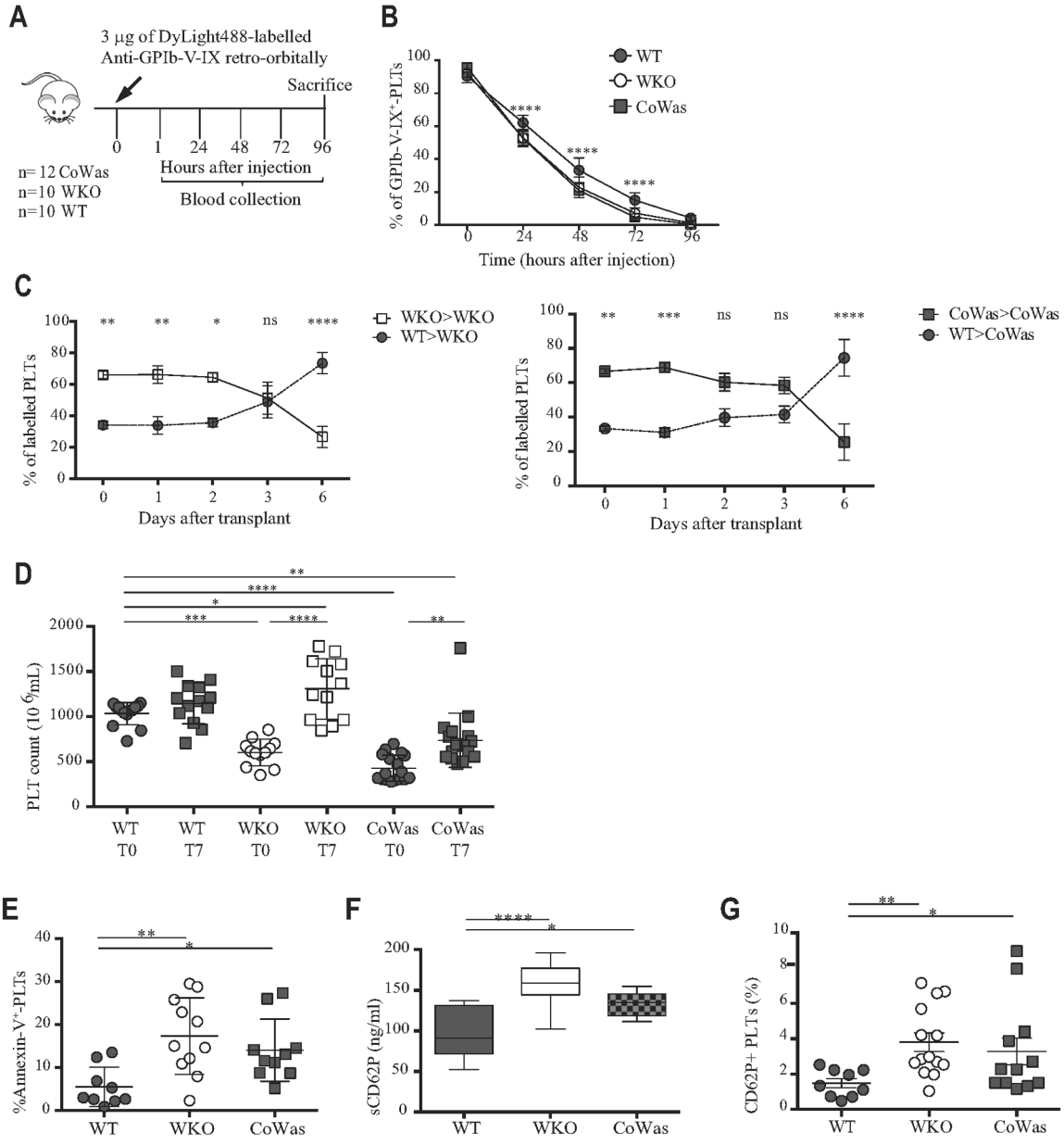

FIG 3.

Peripheral elimination of platelets. **A**, Experimental plan of *in vivo* platelet half-life detection. **B**, Platelet half-life was analyzed as the percentage of GPIb-V-IX^{+} platelets 15 minutes after injection (time 0) and at different time points after injection. **C**, Differentially labeled WT and *Was*$^{-/-}$ platelets have been mixed in a 30%/70% ratio and injected into recipients. *Was*$^{-/-}$ platelets isolated from WKO mice have been transferred to WKO mice *(left panels)*, whereas *Was*$^{-/-}$ platelets isolated form CoWas mice have beentransferred into CoWas mice(*rightpanels*; n = 5). **D**, Platelet counts havebeen evaluatedbefore clodronate liposome injection (T0) and after7 days (T7). **E**, Apoptotic platelets are expressed as Annexin V^{+} platelets. **F**, sCD62P (in nanograms per milliliter) plasma levels (n = 10). **G**, The graph shows CD62P^{+} platelets evaluated by means of flow cytometry and expressed as

a percentage of the total platelet population. In all graphs each *dot* represents a different mouse from 1 (Fig 3, *C*), 2 (Fig 3, *E* and *F*), 3 (Fig 3, *B* and *G*), or 6 (Fig 3, *D*) independent experiments. All graphs report means ± SDs. Fig 3, *C*, reports means ± SEMs. Statistical analysis was performed with 1-way ANOVA(Fig 3, *D*-*F*) or 2-way ANOVA (Fig 3, *B* and *C*) and the Bonferroni postcorrection test. Fig 3, *G*, reports means ± SDs and has been analyzed with the Mann-Whitney test. **P* < .05, ***P* < .005, ****P* < .001, and *****P* < .0001. *ns*, Not significant; *PLT*, platelets.

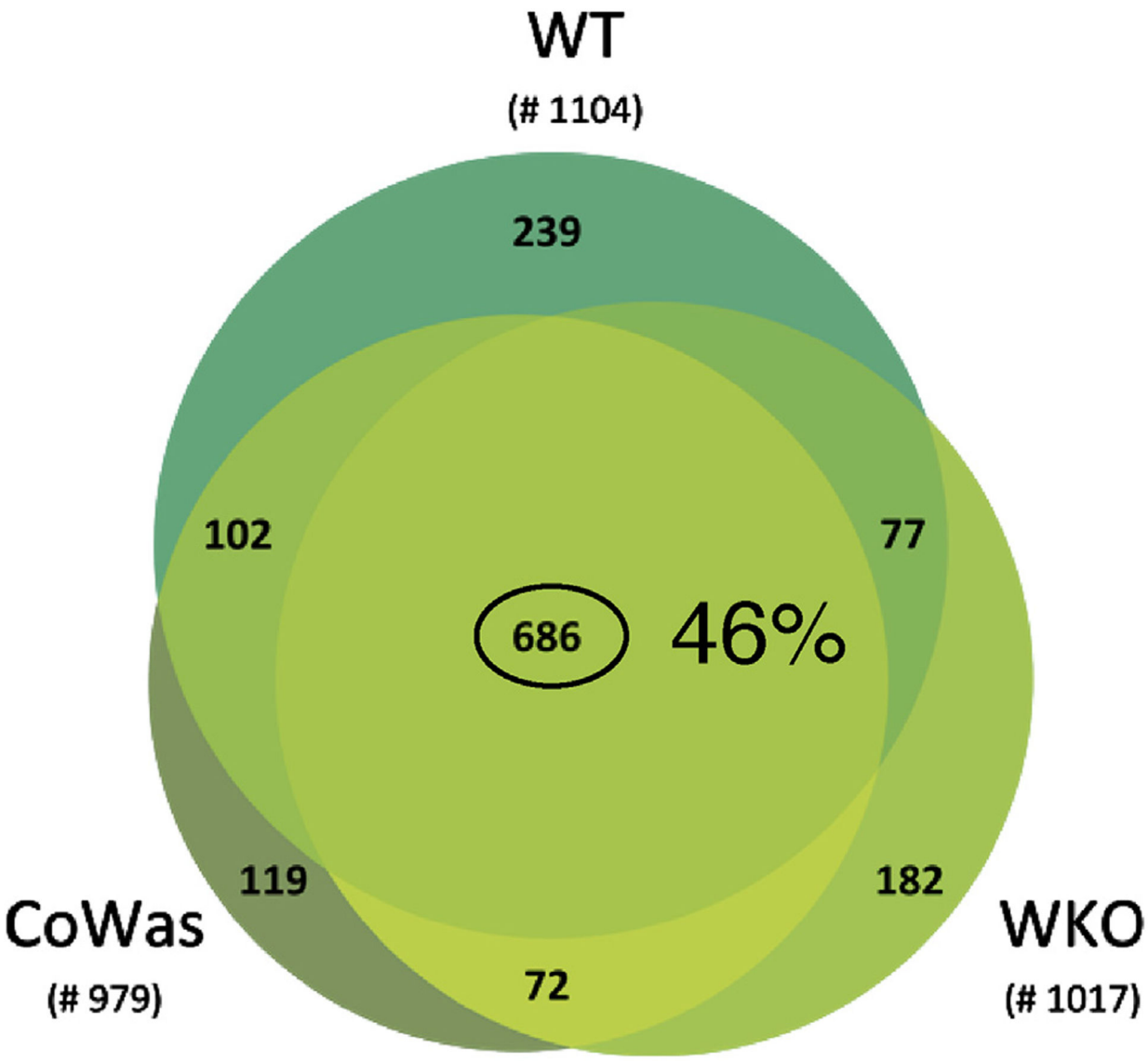

FIG 4.

Proteins identified by using proteomic analysis. Venn diagram of proteins identified in different platelet conditions. The number of total proteins identified was 1104 in WT samples and 1017 and 979 in WKO and CoWas samples, respectively. Six hundred eighty-six proteins (corresponding to 46% of the total protein identified) are shared between mutant and WT animals.

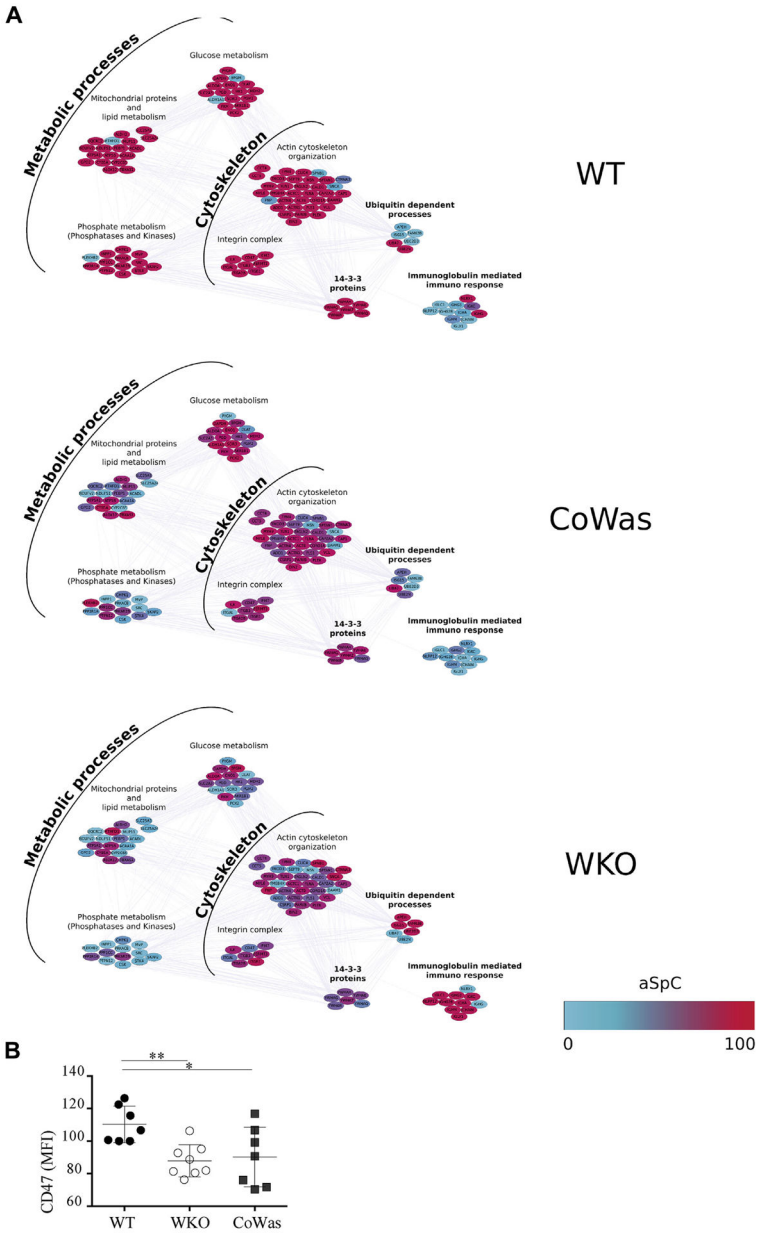

FIG 5.

Different proteomic profile between WT and *Was*$^{-/-}$ platelets. **A**, Focus on the main biological processes identified during proteomic analysis. Proposed clusters were selected by using the BINGO Cytoscape plugin ($P \leq .05$), and color gradients correspond to the normalized average spectral count (*aSpC*; 0–100), where red indicates upregulated proteins, *blue* indicates downregulated proteins, and the shade between *red* and *blue* indicate intermediate level of expression. **B**, The graph shows the CD47 MFI on the CD61^{+} platelet subpopulation. Statistical analysis has been performed with the unpaired *t* test. Each *dot* represents a mouse. *$P < .05$ and **$P < .005$.

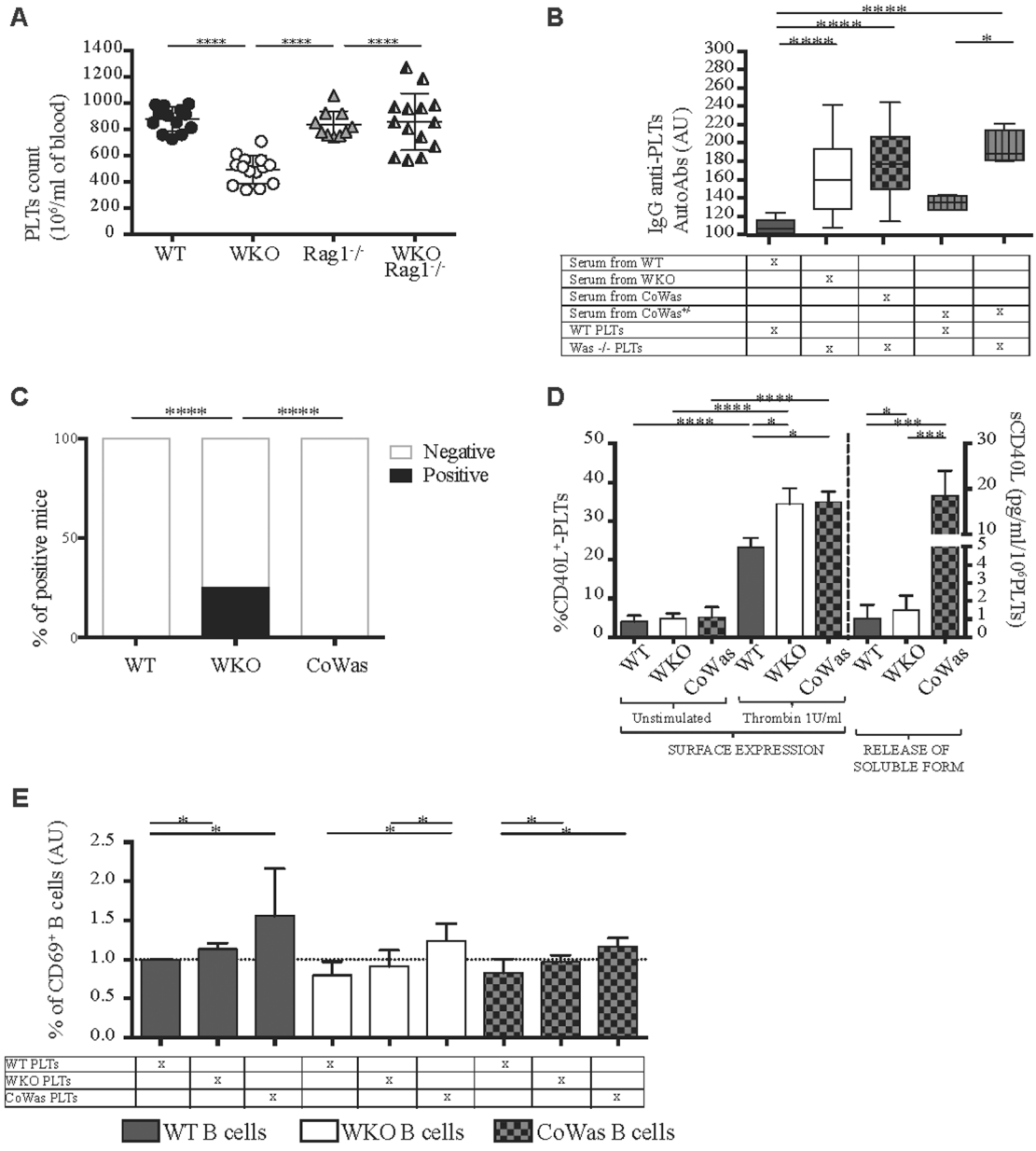

FIG 6.

Interaction between platelets *(PLTs)* and B cells mediated by CD40L. **A**, Platelet counts from age-matched WT, WKO, *Rag1*$^{-/-}$, and WKO- *Rag1*$^{-/-}$ mice are shown. **B**, Evaluation of IgG antiplatelet autoantibodies. ELISA plates were coated with total protein lysates from WT or *Was*$^{-/-}$ platelets and incubated with sera according to the grid presented (n = 23). **C**, The graph represents the percentage of mice positive for the anti-double-stranded DNA autoantibodies. **D**, Percentage of CD40L^{+} platelets was evaluated by using flow cytometry before (n = 10) and after (n = 12) stimulation with thrombin (1 U/mL; *left*); sCD40L was measured in PPP from unwashed platelets isolated from WT (n = 16), WKO (n = 16), and CoWas (n = 5) mice *(right)*. **E**, Resting B cells (CD43^{-} cells) isolated from the spleens of WT, WKO, and CoWas mice were kept in culture for 72 hours with PPP and analyzed by

using flow cytometry for CD69 expression as an activation marker. In Fig 6, *A*, each *dot* represents a different mouse, and means ± SDs are reported. In Fig 6, *B, D*, and *E*, the mean ± SD reported is the result of 2 (Fig 6, *B*) and 4 (Fig 6, *D*) independent experiments. Statistical analysis was performed with 1-way ANOVA and the Bonferroni postcorrection test (Fig 6, *A, B*, and *D, left panel*). In Fig 6, *D*, sCD40L levels *(rightpanel)* are analyzed with the Mann-Whitney test, as well as Fig 6, *D*. Fig 6, *C*, has been analyzed statistically with the χ^2test. **P* <.05, ***P* < .005, ****P* < .001, and *****P* < .0001.

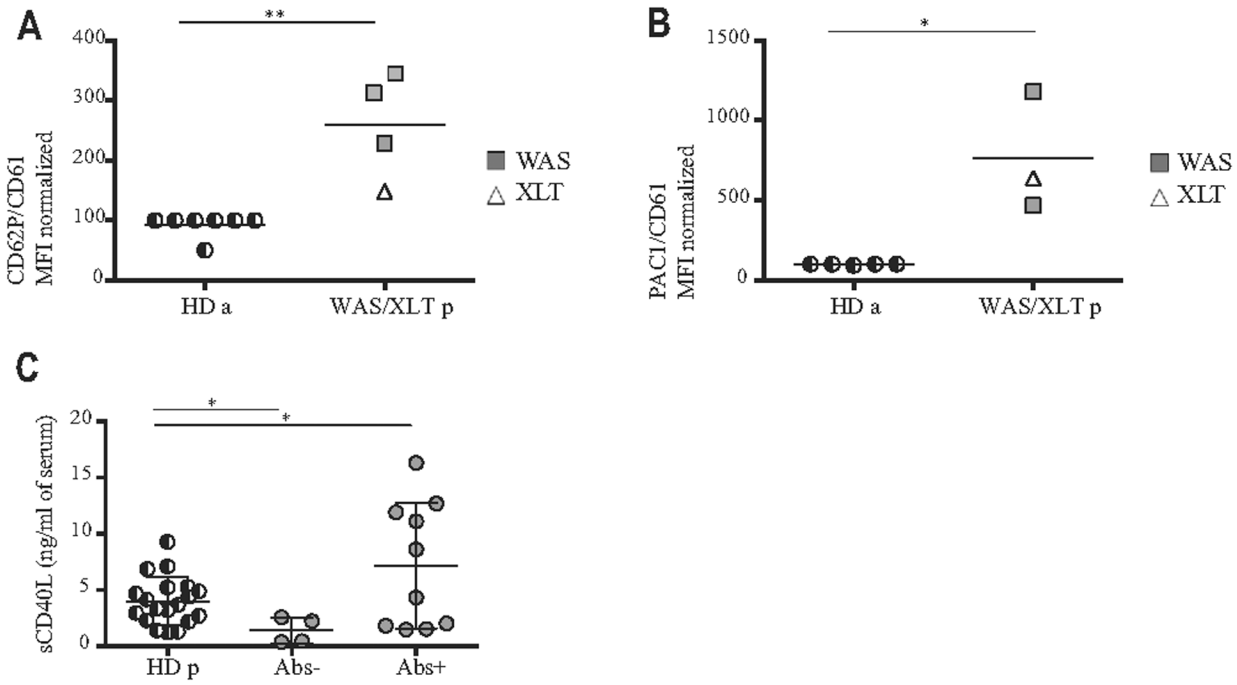

FIG 7.

Activation profile of human platelets. **A** and **B**, Activation profile of human platelets expressed as the MFI ratio of activation markers and CD61 MFI (Fig 7, *A*, for CD62P and Fig 7, *B*, for PAC1). We assigned to the unstimulated adult HD *(HD a)* a value of 100, and we normalized the patients accordingly. In Fig 7, *A*, we analyzed 4 patients (3 with WAS and 1 with XLT) and 7 HDs; in Fig 7, *B*, we analyzed 3 patients (2 with WAS and 1 with XLT) and 5 HDs. **C**, The level of sCD40L in the plasma of patients with WAS/XLT was analyzed by means of ELISA. Patients with WAS/XLT were divided according to the presence or absence of autoantibodies or other clinical signs related to autoimmunity (see Table E3). Pediatric HDs *(HD p)* were tested as control subjects. Fig 7, *A* and *B*, show means analyzed with the Mann-Whitney test. Fig 7, *C*, reports means ± SDs and shows analysis with 1-way ANOVA and the Bonferroni postcorrection test. *$P < .05$ and **$P < .005$.