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Platelets interact with Coxsackieviruses B and have a critical role in the pathogenesis of virus-induced myocarditis

S. NEGROTTO,*[1] C. JAQUENOD DE GIUSTI,†[1] L. RIVADENEYRA,* A. E. URE,† H. A. MENA,* M. SCHATTNER* and R. M. GOMEZ†

*Laboratorio de Trombosis Experimental, Instituto de Medicina Experimental, ANM-CONICET, Buenos Aires; and †Laboratorio de Virus Animales, Instituto de Biotecnología y Biología Molecular, CONICET-UNLP, La Plata, Argentina

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Summary. *Background:* To further understand the role of platelets in the pathogenesis of viral infections we explored platelet interaction with Coxsackieviruses B (CVB) 1 and 3. CVB is a group of viruses that cause the majority of human enterovirus-related viral myocarditis; their receptor (CAR) is expressed on the platelet surface and there is a well-characterized CVB3-induced myocarditis murine model. *Methods:* Human platelets were infected with CVB1 and 3 and viruses were detected in pellets and in supernatants. C57BL/6J mice with or without platelet depletion were inoculated with CVB3 and peripheral blood and heart samples collected at different times post-infection. *Results:* CVB1 and 3 RNA and a capsid protein were detected in infected platelets. Despite the fact that titration assays in Vero cells showed increasing infectivity titers over time, supernatants and pellets from infected platelets showed similar levels, suggesting that platelets were not susceptible to a replicative infectivity cycle. CVB binding was CAR-independent and resulted in P-selectin and phosphatidylserine (PS) exposure. CVB3-infected mice showed a rapid thrombocytopenia that correlated with an increase in platelet PS exposure and platelet-leukocyte aggregates without modification of platelet P-selectin expression or von Willebrand factor levels. Mortality, viremia, heart viral titers and myocarditis were significantly higher in platelet-depleted than normal animals. Type I IFN levels were not changed but IgG levels were lower in infected and platelet-depleted mice. *Conclusions:* Our data reveal that platelets play a critical role in host survival and immune response against CVB3 infection.

Keywords: human enterovirus B; pathogenesis; platelets; P-selectin; thrombocytopenia.

Correspondence: Ricardo M. Gómez, Instituto de Biotecnología y Biología Molecular, Calle 49 y 115, 1900 La Plata, Buenos Aires, Argentina.
Tel.: +54221 425-9223; fax: +54221 422-9777.
E-mail: rmg1426@gmail.com,

[1]These authors contributed equally.

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Introduction

The interaction between viruses and platelets was described more than 50 years ago [1,2]. The first studies were focused on elucidating the mechanisms involved in thrombocytopenia associated with viral infections. More recent studies have shown that platelets have a more active role in the pathogenesis of viral infection. In this regard, it has been described that platelets bind and transport human immunodeficiency virus (HIV) [3] and Dengue virus (DENV) [4], either for engulfing and destruction [5] or to shelter them from the aggression of the host immune system, as has been shown for hepatitis C virus (HCV) [6]. Furthermore, virus-infected platelets can expose P-selectin or phosphatidylserine (PS), promoting their phagocytosis by macrophages [7,8]. In addition, murine lymphocytic choriomeningitis virus (LCMV) infection studies showed that mild hemorrhagic anemia becomes severe and eventually lethal in animals depleted of platelets by a mechanism mediated by type I IFN (α/β) and the failure of an efficient cytotoxic T lymphocyte response for viral clearance [9]. Collectively, these data show that platelet-virus interaction is more complex than initially believed. However, whether platelets contribute to the transport and dissemination of viruses by taking up viruses and/or to the degree of host antiviral defense is yet not clear.

Coxsackieviruses B (CVB) are small, icosahedral non-enveloped single-strand positive-sense RNA viruses [10].

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Based on their molecular characteristics, the CVB group, which includes six serotypes, is currently classified as human enteroviruses B, in the *picornaviridae* family [11]. CVB commonly cause subclinical infections, but occasionally result in a significant disease [12]. These viruses cause the majority of enterovirus-related myocarditis cases [13], a condition that has been linked to dilated cardiomyopathy [14].

Hemorrhage and/or impaired platelet functionality are not associated with CVB human infections. However, these viruses are interesting for the study of platelet-virus interaction because their specific Coxsackie-Adeno receptor (CAR) expression was described on platelets [15], CVB3 infection in mice resembles human infection [16], and the murine model has been extensively used in pathogenesis studies [12,13,17–19]. Therefore, to further understand the role of platelets during viral pathogenesis, in the present study we analyzed *in vitro* and *in vivo* CVB interaction with platelets and found that platelets play a critical role in host survival and immune response against CVB3 infection.

Materials and methods

Cells

Vero cells (American Type Culture Collection, Manassas, VA, USA) were maintained as monolayers in minimal essential medium (MEM), supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 25.5 mM sodium bicarbonate and 50 mg mL^{-1} gentamicin.

Viruses and antibodies

Two recombinant CVB strains were used: a myocarditic CVB3 (H3) [20] and a non-myocarditic CVB1 strain [21]. Viral stocks, propagation in Vero cells and infectivity titration have been previously described [22]. The antibodies (Abs) against viral antigens and primers have been used before [23]. The blocking antiserum against CAR was generously provided by Dr J.M. Bergelson, University of Pennsylvania, USA. Eptifibatide and anti CD42b (clone HIP1) blocking antibodies were from Santa Cruz (Dallas, TX, USA) and Biolegend (San Diego, CA, USA), respectively. The antiserum against platelets have been described before [24] and was a generous gift from Dr M. Isturiz, IMEX, Argentina.

Human platelets

Blood samples were obtained from voluntary healthy donors. The study was performed according to institutional guidelines and the approval of the Institutional Ethics Committee of the National Academy of Medicine and written consent from all the subjects. Platelet-rich plasma (PRP) was obtained from anticoagulated blood (sodium citrate 3.8%). Platelets were washed as previously described [25] and resuspended in Tyrode's buffer at a concentration of 4×10^8 mL^{-1}. $CaCl_2$ (1 mM) was added 1 min before platelet stimulation. Considering that there was a white cell level of 0.01% in our platelet samples, we further purified the samples using the leukocyte depletion filter system [26].

Experimental design of *in-vitro* assays

PRP were infected with the CVB1 or CVB3 strains at a MOI of 10 for the indicated time at 37 °C using Biosafety Level 2 practices, equipment and facility design, as recommended by the Public Health Agency of Canada (http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/msds44e-eng.php). After three exhaustive washes (220 × *g*, room temperature, 10 min) to ensure viral removal, platelets were resuspended in RPMI/5% FCS and maintained in a humidified incubator at 37 °C until use. For some experiments, mock and CVB-infected platelets were stimulated (or not) for 5 min with thrombin (0.05 U ml^{-1}) and supernatants and pellets were collected at different hours post-infection (hpi) and kept at −80 °C until assayed.

To identify the receptors mediating platelet-CVB interaction, platelets were preincubated for 30 min with blocking Ab against CAR, CD42b (40 μg mL^{-1}) or with eptifibatide (10 μg mL^{-1}). Under these conditions, eptifibatide completely blocked thrombin-induced fibrinogen binding and anti-CD42b blocking antibody exerted a 100% inhibition of the FITC-conjugated anti-CD42b binding (clone HIP1, BD Pharmingen, San Diego, CA, USA) (data not shown).

RT-PCR and real time PCR

RT-PCR was performed as previously described [22]. Specific primers for CVB3 and actin were used (CVB3 fwd: CGGCCCCTGAATGCGGCTAA; CVB3 rev: GAAACA CGGACACCCAAAGTA; Actin fwd: CGTCATCCATG GCGAACTG; Actin rev: GCTTCTTTGCAGCTCCTTC GT). Real-time PCR (qPCR) for type I IFN was performed using specific primers (IFN-Iα fwd: TCCATGAG ATGATCCAGCAG; IFN-Iα rev: ATTTCTGCTCTGAC AACCTCCC; IFN-Iβ fwd: GCTCTCCTGTTGTGCTTC TCCAC; IFN-Iβ rev: CAATAGTCTCATTCCAGCCAG TGC), following protocols that were described previously [26].

Immunofluorescence

Cells fixed with 1% paraformaldehyde were centrifuged and permeabilized with 0.1% Triton X-100. After blocking with 6% BSA, cells were incubated overnight with the primary Ab against enterovirus VP1 protein followed by Alexa 488-conjugated secondary Abs (Life Technologies, Grand Island, NY, USA). Slides were analyzed under confocal microscopy (Olympus FV-1000, Tokyo, Japan).

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Immunoblotting

Platelets ($1–2 \times 10^9$ mL^{-1}) were lysed in loading buffer (62.5 nM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue and 5% 2-mercaptoethanol) and subjected to electrophoresis on a 12% SDS-PAGE and electrotransferred to nitrocellulose membranes (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK). After blocking, membranes were incubated overnight at 4 °C with a primary monoclonal Ab against VP1, followed by an HRP-linked secondary Ab (Dako, Glostrup, Denmark) for 1 h at 22 °C. Protein bands were visualized by ECL reaction and optical density was semi-quantitated using Gel-Pro analyzer 3.1 software (Media Cybernetics Inc, Rockville, MD, USA). Values from blot re-probes against actin were used for normalization of data for protein loads.

Flow cytometry

To evaluate P-selectin expression, mock- or CVB-infected platelets were fixed and stained with an FITC-conjugated CD62P (anti-P-selectin Ab, clone AK-4 and RB40.34, anti-human and anti-mouse, respectively) or a matched IgG isotype as a negative control (BD Biosciences, San Jose, CA, USA) for 10 min at room temperature. PS expression was determined by FITC-annexin V binding for 15 min at room temperature in the dark using a commercial kit (BD Pharmingen). Flow cytometry analysis was performed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) using FCS Express V3 software (De Novo Software, Los Angeles, CA, USA).

Animal studies

Eight-week-old male and female C57BL/6J mice, originally purchased from Jackson Laboratory, Bar Harbor, ME, USA, were housed in a controlled environment with free access to water and a standard diet. Animal studies were performed in accordance with the regulations of the Argentinian Council for Animal Care and with the approval of the Institutional Animal Care Committee.

Mice were infected intraperitoneally with 1×10^4 UFP of a myocarditic variant of CVB3 and blood sampling was performed via the retro-orbital plexus and collected in EDTA (2 mM) at different hpi. P-selectin and PS expression in platelets were measured after red cell lysis with an NH_4Cl solution, as described above. Mixed aggregates were tested by flow cytometry in fresh blood drawn at 1 hpi by double-staining with FITC-CD41 (BD Pharmingen) and PE-Ly-6G Abs (Biolegend, San Diego, CA, USA) or the corresponding isotypes for 15 min at 22 °C. After fixation with 1% PFA, analysis was performed on granulocytes only by gating this population based on its FSS and SSC features. von Willibrand factor (VWF) levels were quantified in plasma by ELISA [27].

For platelet depletion, animals were inoculated intraperitoneally with PBS or with 15 μL (single dose) of a specific polyclonal antiserum against platelets every 24 h. Blood cell counts were assessed on an automated analyzer (Abacus Jr Vet, Vienna, Austria). Platelet-depleted and non-depleted mock-infected animals were used as negative controls. Mice were euthanized at 3, 6 and 12 days post-infection (dpi), and blood and heart samples were collected. Heart homogenates were split into three pieces, weighted and processed to analyze levels of infectivity virus by plaque assay, as well as for routine histology as previously described [21,22]. Myocarditis was scored as previously described [22]. IgG levels were quantified in plasma by ELISA. Type I IFN levels were determined in heart homogenates using an already described qPCR procedure with minor modifications [28].

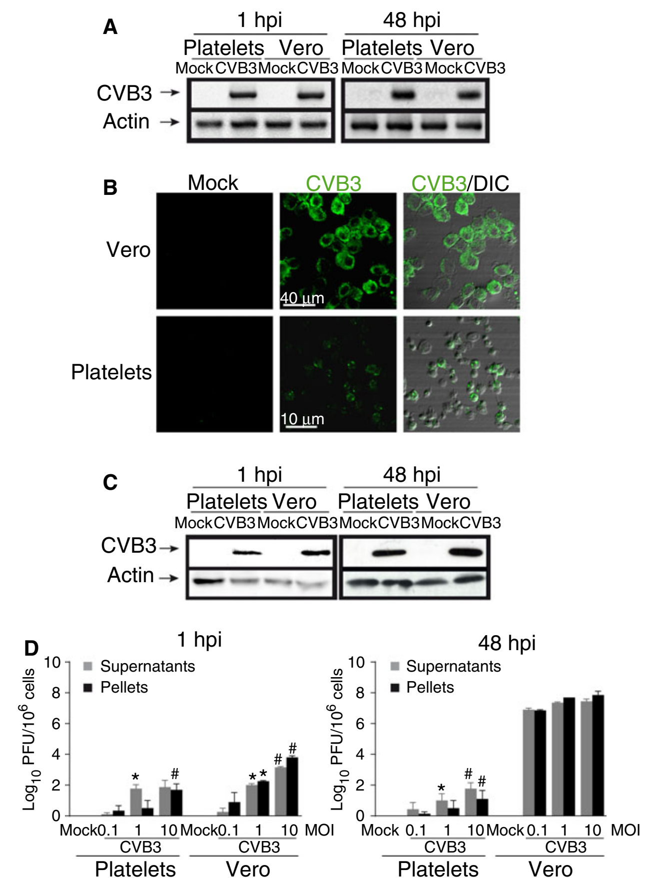

Fig. 1. Coxsackieviruses B (CVB) interact with platelets. Human washed platelets and Vero cells were infected or not with CVB3 and the presence of viral particles was detected at 1 and 48 hours post-infection (hpi). (A) CVB3 mRNA was detected by RT-PCR (n = 3). (B) The expression of the enterovirus protein VP1 (green) was determined by immunofluorescence (n = 4). Differential interference contrast (DIC) and fluorescence images were analyzed by confocal microscopy. Original magnification 60×; zoom factor 4× in platelet images. (C) VP1 expression was confirmed in cell lysates by immunoblotting (n = 3). (D) Platelets were infected with CVB3 at different multiplicities of infection (MOIs) and plaque forming units (PFU) in supernatants and pellets were quantified by the infectivity titration assay on Vero cells (n = 5). *$P < 0.05$ vs. Mock, one-way ANOVA., $^{\#}P < 0.05$ vs. MOI^{-1}

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Statistical analysis

Results are expressed as means ± SEM. Each n represents a different donor. The Student's paired *t*-test or one- or two-way analysis of variance (ANOVA) followed by Bonferroni test were employed to determine the significance of differences between the groups as indicated in each figure legend. A P value lower than 0.05 was considered to be statistically significant.

Results

Platelets interact with CVB

We initially explored the CVB-platelet interaction by incubating platelets with either CVB1 or CVB3 strains; the presence of viral RNA, antigen and infectious viral particles were determined at 1 and 48 hpi. An extensive washing was performed prior to virus detection to ensure removal of the exogenous inoculated virus and infected Vero cells were used as positive controls. Figure 1(A) shows the presence of viral RNA by RT-PCR. The CVB3 capsid protein in some platelets and Vero cells was detected by immunofluorescence (Fig. 1B). These data were confirmed by immunoblotting assays (Fig. 1C). Moreover, titration assays with Vero cells showed that infectious viral particles were detected in supernatants and pellets from CVB3-infected platelets, where the increasing MOIs, but not time, correlated with infectivity titers (Fig. 1D). Similar results were obtained with CVB1 (data not shown). Our results indicate that platelet-CVB interaction occurs at early time-points (1 h) and persists for at least 48 h.

CVB-platelet interaction is not mediated by CAR and does not result in virus replication

As it has been postulated that human platelets express CAR, we next investigated whether the observed platelet-CVB interaction involved this receptor by using a CAR-blocking Ab during infection. Results showed that

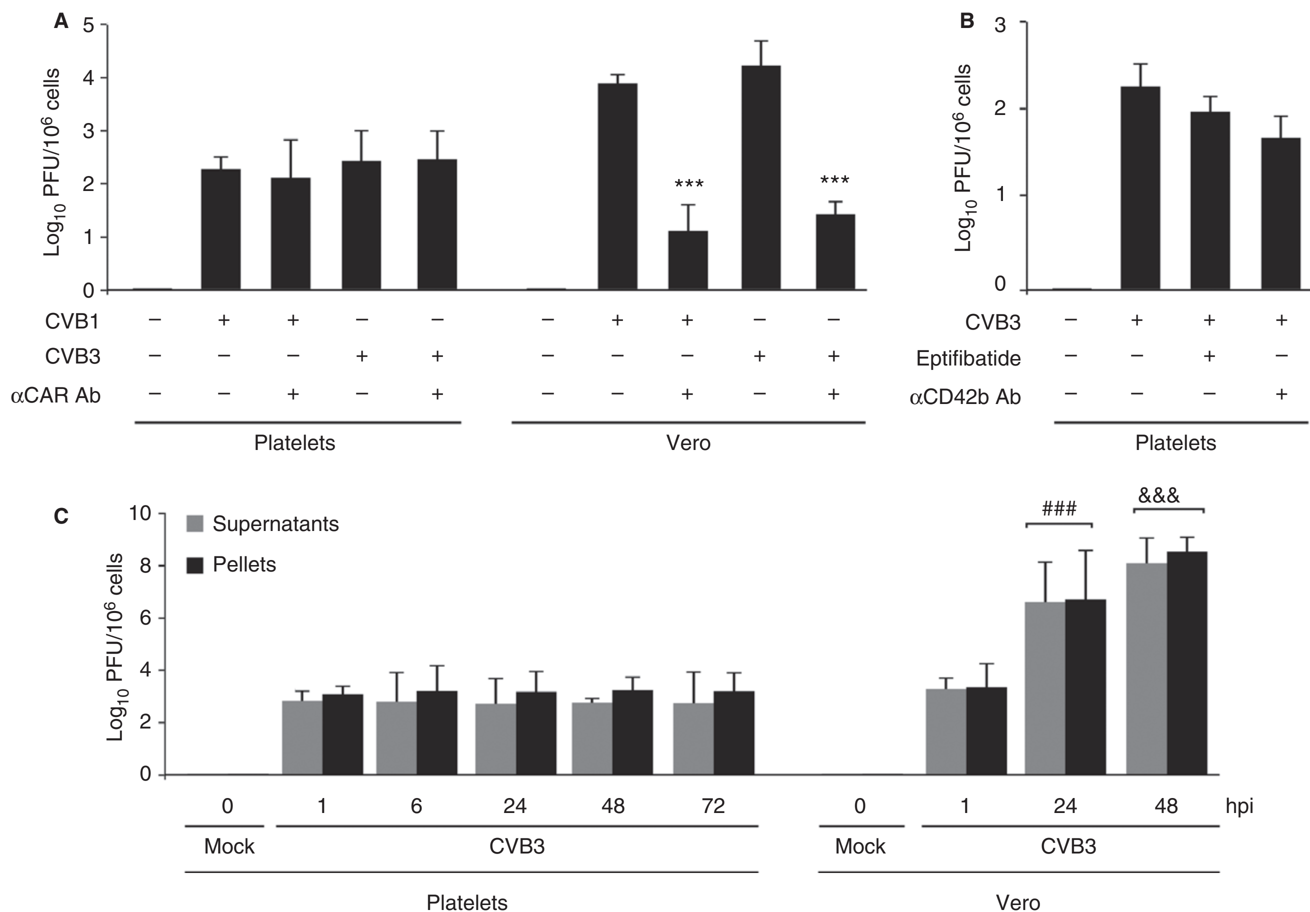

Fig. 2. Coxsackieviruses B (CVB)-platelet interaction is Coxsackie-Adeno receptor (CAR) independent and does not result in virus replication. (A) Human washed platelets and Vero cells were infected or not with CVB1 or CVB3 in the absence or presence of a CAR blocking antibody (αCAR Ab) and plaque forming units (PFU) in cell pellets were quantified by the infectivity titration assay on Vero cells at 1 hour post-infection (hpi) ($n = 5$). ***$P < 0.001$ vs. CVB-infected cells without αCAR Ab, one-way ANOVA. (B) Platelets were infected or not with CVB3 in the absence or presence of eptifibatide or CD42b blocking antibody (αCD42b Ab) and PFU in cell pellets were quantified by the infectivity titration assay on Vero cells at 1 hpi ($n = 3$). (C) PFU in platelet and Vero cell supernatants and pellets were quantified at indicated time-points by the infectivity titration assay on Vero cells ($n = 4$). ###$P < 0.001$ vs. 1 hpi, &&&$P < 0.001$ vs. 24 hpi, one-way ANOVA.

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while CVB1 or CVB3 binding to Vero cells was significantly inhibited by the CAR-blocking Ab, this inhibitory effect was not observed in platelets (Fig. 2A), suggesting that CVB-platelet interaction is not mediated by CAR.

In addition, when major platelet glycoproteins (GP) $\alpha_{IIb}\beta_3$ integrin and GPIb were blocked with eptifibatide or the neutralizing antibody CD42b, respectively, infectivity titers in pellets at 1 hpi were moderately reduced by 12 and 25%, respectively (Fig. 2B). Platelets are anucleated cells; however, they have the machinery required for RNA translation [29], which potentially enables virus replication. Thus, in order to determine whether CVB replicate in platelets, infectivity titers in pellets or supernatants were followed over time. As similar results were obtained with CVB1 and CVB3, our subsequent studies were conducted with CVB3 only. Although infectious CVB3 was detected in both platelet supernatants and pellets as late as 72 hpi, titer levels were similar between 1 to 72 hpi (Fig. 2C). In contrast, and as expected, Vero-infected supernatants and pellets showed a significant increase in infectivity titer levels from 1 to 48 hpi (Fig. 2C), with almost 100% of the monolayer showing a cytopathic effect. These results suggested that, although the virus replicated in Vero cells amplifying its number over time, the CVB associated with platelets appeared to be the result of viral infectious particles that were bound to the platelet surface or released to supernatants rather than due to replication.

CVB binding to human platelets up-regulates P-selectin and PS expression

As it has been demonstrated that some viruses induce several platelet activation responses, including P-selectin, PS and CD40L expression as well as platelet-leukocyte aggregates, [8,30,31], we next explored the expression of P-selectin and PS as the main mediators of platelet-leukocyte interactions and platelet apoptosis. CVB3 binding to platelets resulted in an increased P-selectin expression and PS exposure levels after 1 hpi (Fig. 3A), which remained unchanged after 24 or 48 hpi; this suggests that the augmented platelet reactivity was associated with the initial contact of platelets with CVB. These experiments were also performed with murine platelets, which displayed higher levels of PS, but not P-selectin, after CVB infection *in vitro* (Fig. 3B).

Moreover, we found that the pan caspase inhibitor Z-VAD-FMK (20 μM) did not block PS exposure, suggesting that platelet apoptosis is not involved (data not shown).

In vivo *murine CVB3 infection fails to modify P-selectin or VWF levels but induces thrombocytopenia, heterotypic aggregates formation and platelet PS exposure*

To further elucidate the relevance of the *in vitro* data, we next infected mice with the pathogenic variant CVB3. CVB-platelet interaction was also demonstrated in the murine model, as infectious viral particles were detected in platelets from CVB-infected mice at 48 hpi (10–100 PFU / 10^6 platelets, $n = 4$). CVB infection also induced a rapid increase in platelet-granulocyte aggregate formation (Fig. 4A). Although it has been reported that adenoviruses induce heterotypic aggregate formation in a P-selectin- and/or VWF-dependent manner [8], our data showed that neither P-selectin (Fig. 4B) nor VWF (Fig. 4C) were significantly augmented by CVB3, even after 48 hpi. Remarkably, we did observe a marked increase in PS exposure that peaked at 6 hpi and remained up-regulated at 48 hpi (Fig. 4D). Moreover, these effects were accompanied by a significant reduction in platelet count (Fig. 4E).

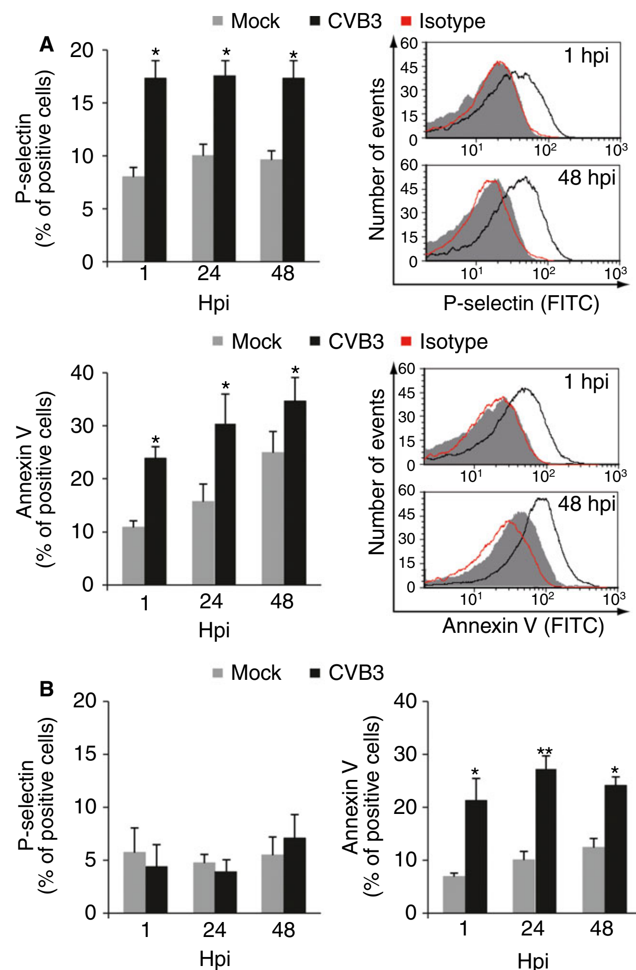

Fig. 3. P-selectin and phosphatidylserine (PS) expression at the platelet surface are up-regulated by Coxsackievirus (CVB). Human (A) or murine (B) washed platelets were infected or not with CVB3. P-selectin and PS exposure levels were determined at the indicated time-points by flow cytometry after cell staining with FITC-CD62P or FITC-annexin V, respectively ($n = 6$). Histograms represent one of six independent experiments. $^*P < 0.05$ and $^{**}P < 0.01$ vs. Mock, two-way ANOVA.

Platelets are critical mediators of viral clearance and survival

To analyze the role of platelets in viral clearance, we measured the levels of viremia in normal or platelet-

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depleted animals. Treatment of animals with an antiserum against platelets [24] induced a drop in the platelet counts below 40 000 μL^{-1}, as soon as 4 h post-treatment (Figure S1). Figure 5(A) shows that viremia was detected as early as 1 h after CVB3 infection and was maximal at 48 hpi. Viremia values of platelet-depleted mice were significantly higher than those of animals with normal platelet counts at 72 hpi (Fig. 5A). These data correlated with a drop in platelet count, which showed the lowest values at 72 hpi (Fig. 5B). Of note, white blood cell count was not affected by platelet depletion, although it was modified by CVB infection (Table S1), and no signs of bleeding were found in either the brain or intestine at that time-point (Figure S2). Bone marrow histology showed that megakaryocyte number and size were moderately increased by CVB infection, antiserum inoculation or the combination of both after 72 hpi (Figure S3). Concomitantly with the absence of viremia at 6 dpi, platelet numbers in non-depleted infected animals returned to basal values on that day (data not shown). We next evaluated the relevance of platelets to the survival of infected mice and found that while mortality was 25% in CVB3-infected mice at 3 dpi, 75% of the infected and platelet-depleted mice were dead. No further deaths were observed after 3 dpi (Fig. 5C).

Platelet depletion increases severity of CVB3-induced myocarditis

Having determined the important role of platelets in viral clearance and survival, we next explored the influence of platelets on the severity of myocarditis induced by CVB3. Infectivity assays of heart tissue homogenates showed significantly higher and persistent viral titers in CVB3-infected and platelet-depleted mice (Fig. 6A). In contrast, the levels of mRNA of both type I IFN α and β were similar in the two groups of animals at 3 dpi (Fig. 6B). Analysis of histopathology showed multifocal acute myocarditis in all infected animals. However, the number and extent of lesions were clearly higher in hearts of the infected and platelet-depleted group at 6 dpi (Fig. 6C). These data show that platelet depletion produces an uncontrolled viral infection that results in a more severe myocarditis.

To analyze the relevance of platelets in the adaptive immune response, we assessed IgG serum levels after 12 dpi. We found that IgG levels were significantly lower in platelet-depleted animals compared with non-depleted ones (Fig. 6D).

Discussion

Besides their role in hemostasis, platelets have immunological functions and participate in the interaction between pathogens and host defense [32]. To obtain deeper insight into the role of platelets during viral infection, we have analyzed the interaction of platelets with CVB in this study. Our *in vitro* investigations showed that CVB1 and 3 interact with human platelets and, although the virus does not replicate in platelets, trigger P-selectin and PS membrane expression.

Our initial detection of infectious CVB associated with both platelet supernatants and pellets prompted us to explore whether the association was mediated by the CVB receptor CAR. While treatment with the anti-CAR blocking Ab significantly reduced (> 2 logs) the titers in Vero cells, viral titers associated with platelet supernatants or pellets were not changed following preincubation with the anti-CAR Ab, strongly suggesting that the association between CVB and platelets was CAR-independent. Of note, the presence of CAR on the platelet surface is still controversial, because it has been shown to be absent [33], present in all platelets [8] or restricted to a sub-fraction (3%) of the platelets [15]. Although some CVB interact with the decay-accelerating factor (DAF) [34,35], which is a complement regulatory protein that is expressed commonly on most cell surfaces, including human platelets [36], our previous studies showing that the CVB3 variant used was not blocked by soluble recombinant human DAF [22] or by blocking antisera, as reported by others [37], strongly suggest that DAF was not involved. Although we observed a moderate reduction when platelet glycoproteins (GP) $\alpha_{IIb}\beta_3$ integrin and GPIb were blocked, the reported association of platelets with many different viruses such as Herpes, Vaccinia, Hanta, Echo, HIV or DENV, points out that platelets can bind viruses in a receptor-independent way, as has been proven for HCV [3,4,6,30,38–42]. Our data did not characterize whether CVB were inside or stuck on the surface. However, the finding of infectious virus in the pellets as well as in the platelet's supernatants allows us to speculate that

Fig. 4. Coxsackievirus (CVB) infection *in vivo* induces platelet activation responses. Blood samples were collected from Mock- or CVB3-infected mice at the indicated time-points and red cell lysis was performed (n = 6). (A) Cells were double-stained with FITC-CD41 and PE-Ly6G antibodies against murine platelets and granulocytes, respectively. Heterotypic aggregates (CD41 + /Ly6G+ cell population) were analyzed at 1 hour post-infection (hpi) by flow cytometry by gating the granulocyte population in FSC vs. SSC dot plots. FL1 vs. FL2 dot plots represent one of six independent experiments and quadrant markers to define a negative population were set up based on the isotype-related fluorescence. $^{**}P < 0.01$ vs. Mock, Student's t-test. (B) Cells were stained with an anti-murine FITC-CD62P antibody and P-selectin expression levels were analyzed by flow cytometry. (C) VWF plasma levels were quantified by ELISA. (D) Cells were incubated with FITC-annexin V and phosphatidylserine exposure was measured by flow cytometry. (E) Platelet counts were performed in an automated analyzer. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. Mock, two-way ANOVA.

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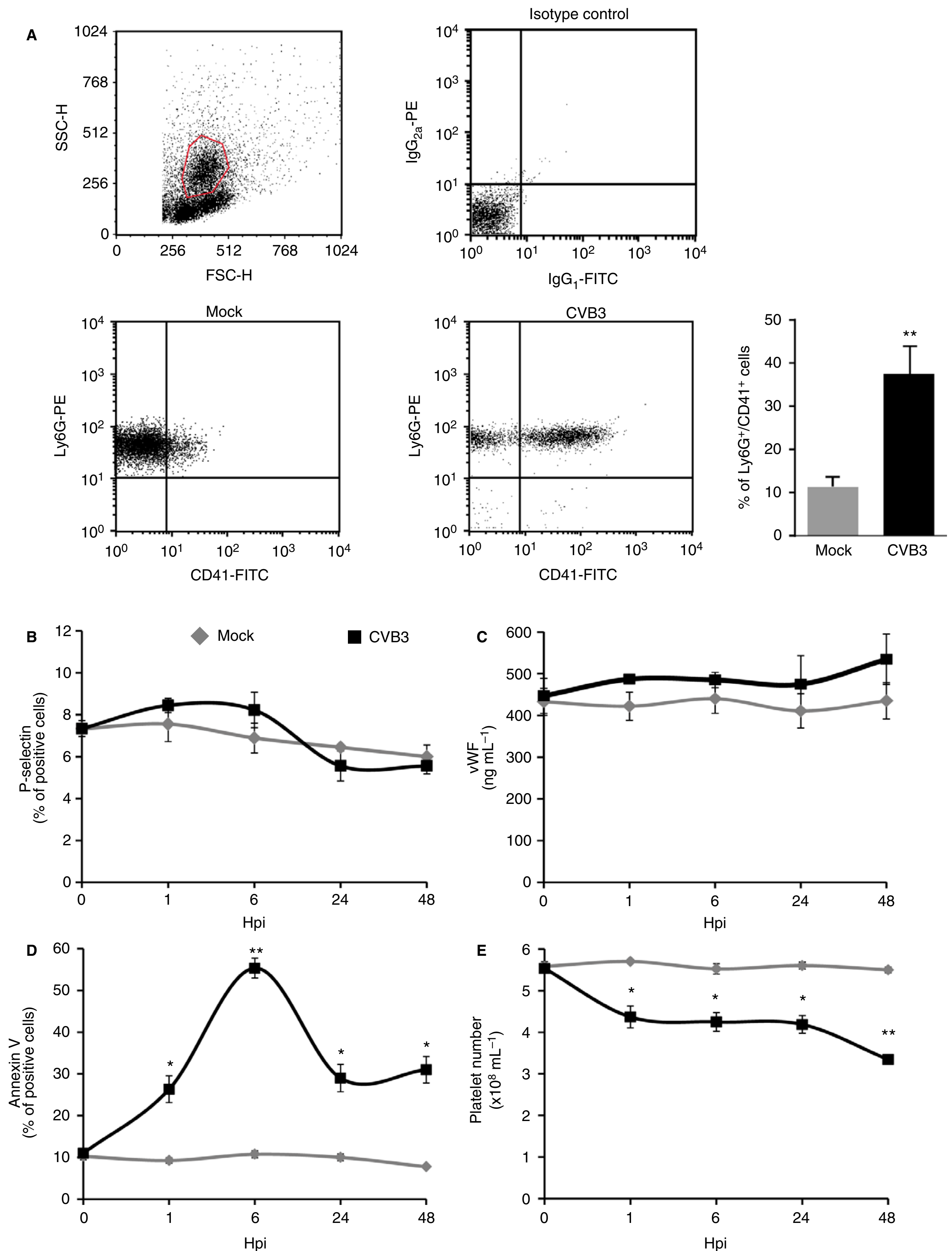

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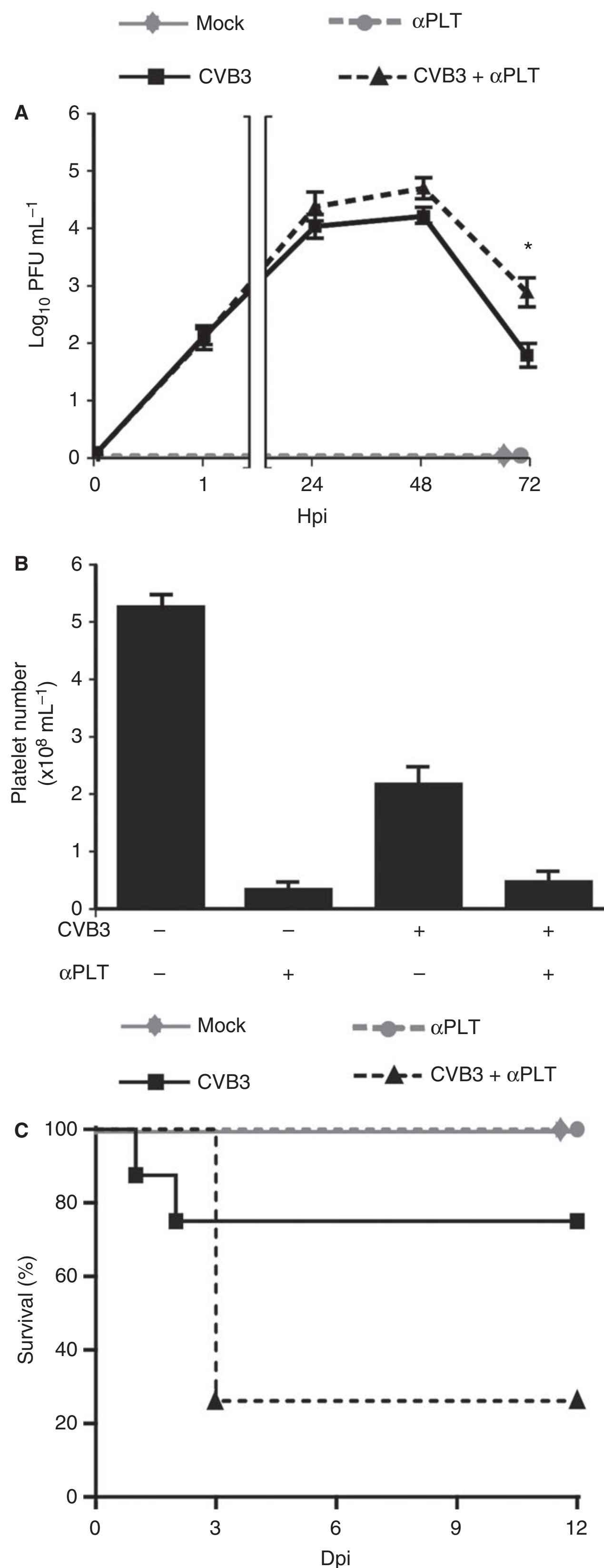

Fig. 5. Platelet depletion decreases viral clearance and survival in Coxsackievirus (CVB)-infected mice. CVB3-infected mice were treated or not with antiserum against murine platelets (αPLT) and blood samples were collected at the indicated time-points (n = 9). (A) Viremia (PFU mL^{-1} of plasma) was determined by the infectivity titration assay on Vero cells. *$P < 0.05$ vs. CVB3, two-way ANOVA. (B) Platelet counts were performed in an automated analyzer at 72 days post-infection (dpi). (C) Percentage of survival was registered in all groups.

the open canalicular system might play a role in the harboring and release of CVB, as has been shown for other viruses [5,30].

Although platelets have the molecular machinery required to translate RNA [29], a critical step but not the only one that may enable virus replication, to the best of our knowledge, no studies have been published demonstrating that platelets promote or fail to support viral replication. Although we found viral particles in the supernatants and pellets of CVB-infected platelets, the observation that their levels did not increase over time strongly suggests that CVB do not replicate in platelets. Nevertheless, we cannot exclude the possibility that the *in vitro* strategies used here were not sensitive enough or that this event can take place *in vivo*. Further studies are therefore required to elucidate these issues.

CVB binding to platelets induces activation because P-selectin and PS levels in the surface membrane were higher in human platelets incubated with virus compared with the mock-infected controls. Regarding murine platelets, we here show that they displayed higher levels of PS, but not P-selectin, after CVB infection *in vitro*. Interestingly, these data were different to what we observed in human platelets but similar to the *in vivo* findings. Up-regulation of these platelet molecules is not restricted to CVB. For instance, the human adenovirus group C serotype 5 is internalized by platelets in an alphaIIb-beta3 integrin-independent manner and induces P-selectin expression, platelet aggregation and the formation of platelet-leukocyte aggregates [8,30]. DENV-2 exposure can also activate platelets with an increase in P-selectin expression and fibrinogen-binding [31] and also induces the apoptosis of platelets via up-regulation of PS and caspases [7,43]. In this regard, we found that PS exposure upon CVB infection was not blocked by the pan caspase inhibitor Z-VAD-FMK, suggesting that CVB fail to induce platelet apoptosis. Of note, induction of platelet activation seems to be restricted to some viruses, because the Japanese encephalitis virus, although able to bind platelets, does not promote activation or morphological changes [31].

To further understand these *in vitro* data, we analyzed the role of platelets in the pathogenesis of CVB3-infected mice. Our *in vivo* studies show that CVB3 infection triggered platelet PS but not P-selectin expression or VWF release. These effects were accompanied by an increase in the formation of platelet-leukocyte aggregation and thrombocytopenia. The reasons for the differences between *in vitro* and *in vivo* studies regarding P-selectin expression are not clear, but different species

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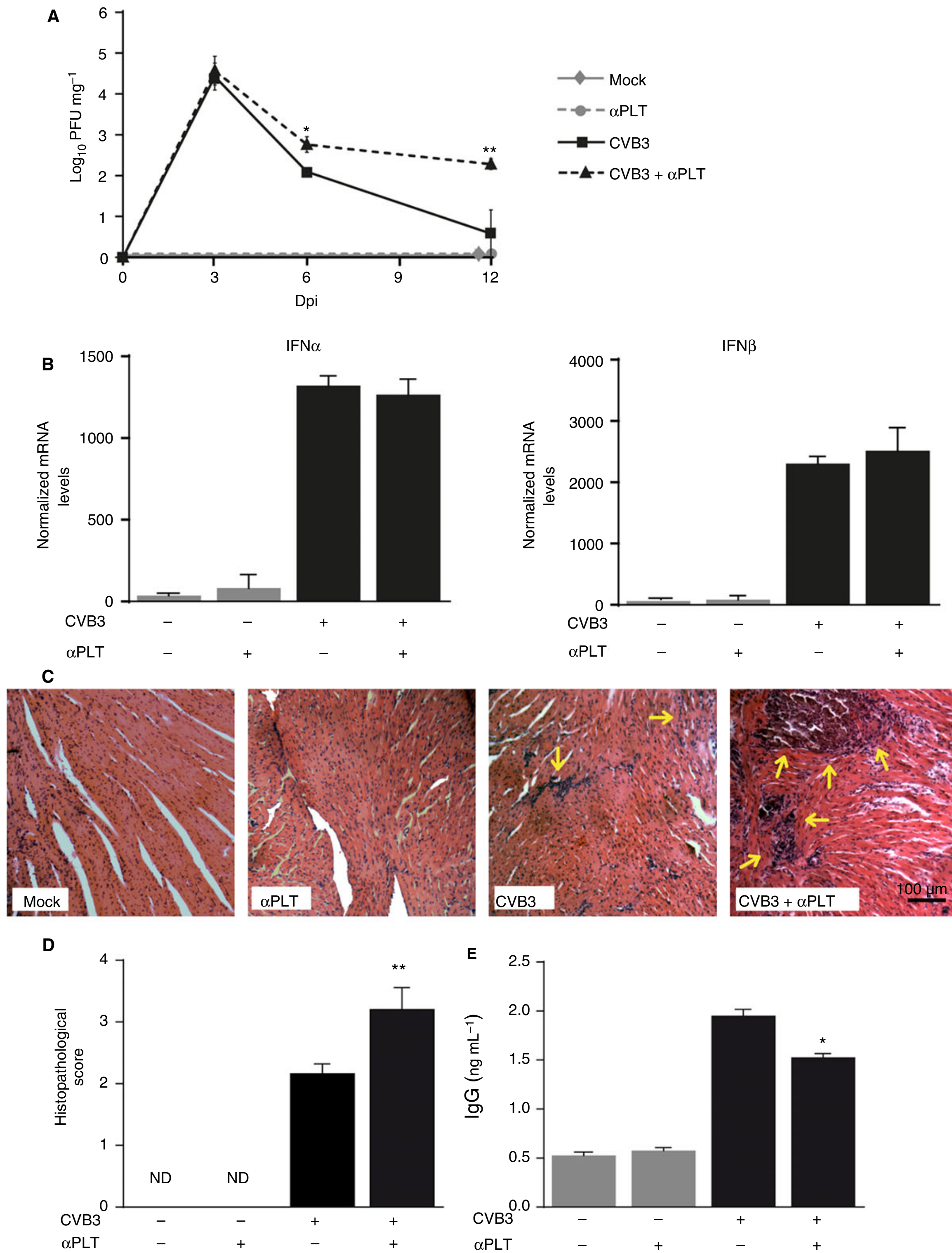

Fig. 6. Platelet depletion increases severity of Coxsackievirus (CVB)-induced myocarditis. CVB3-infected mice were treated or not with antiserum against murine platelets (αPLT) and hearts and blood samples were collected at indicated time-points (n = 6). (A) Plaque forming units (PFU) in heart tissue homogenates were determined by the infectivity titration assay on Vero cells. *$P < 0.05$ and **$P < 0.01$ vs. CVB3, two-way ANOVA. (B) Type I IFN mRNA levels were measured in heart tissue homogenates by qPCR at 3 days post-infection (dpi). (C) Representative images of hematoxylin/eosin stained sections from murine hearts collected at 6 dpi (original magnification 100x). Arrows indicate myocarditic lesions. (D) Blinded histopathological scoring of myocarditis lesions was performed by optical microscopic examination of cardiac sections. Results are expressed in arbitrary units (AU). *$P < 0.05$ vs. CVB3, one-way ANOVA. (E) IgG serum levels were quantified by ELISA at 12 dpi. *$P < 0.05$ vs. CVB3, one-way ANOVA.

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involved in each condition or the presence of plasma components, could be some of them. Thrombocytopenia is a common feature of viral infections. Among the different mechanisms proposed, the formation of mixed platelet-leukocyte aggregates in adenovirus infection [8] as well as platelet apoptosis and macrophage phagocytosis due to PS expression in DENV infections have been shown [43]. Our data confirmed that both events occur in CVB-infected mice, making them both plausible causes of the reduced platelet count. The formation of platelet-mixed aggregates independent of platelet P-selectin expression or VWF was somewhat unexpected, because both molecules were reported to mediate this process after adenovirus infection [8]. These discrepancies could be associated with the different type of virus used in each study.

Of note, a recent study published during the preparation of the current manuscript reported that infection with the single stranded RNA (ssRNA) encephalomyocarditis virus (EMCV) rapidly reduces platelet counts through the formation of large platelet-neutrophil aggregates. The mechanism mediating platelet granule release, the translocation of P-selectin to the cell surface, and a consequent increase in platelet-neutrophil adhesion involved the toll-like receptor 7 (TLR7), which recognizes ssRNA. Interestingly, the authors also showed that not all platelets interacting with white blood cells were P-selectin positive and that P-selectin KO mice still retained the ability to interact with the neutrophil population after TLR7 stimulation, although to a lesser degree [42]. These data clearly indicate that not only P-selectin but also other cell adhesion molecules support platelet-leukocyte interactions.

Our studies in CVB3-infected and platelet-depleted mice showed higher mortality rates, viremia levels, heart viral titers and myocarditis severity, similar type I IFN mRNA levels but decreased IgG serum levels compared with CVB3-infected mice. In previous studies using the Armstrong (acute) strain of LCMV, platelet depletion has been associated with higher mortality, a failed cytotoxic T lymphocyte response and impaired LCMV clearance by a mechanism involving virus-induced type I IFN (α/β), even when depletion was extreme [9] or partial [44]. Increased mortality was also observed after EMCV infection of platelet-depleted mice by a mechanism involving platelets' TLR7 and neutrophil-platelet aggregates [42]. Our study confirmed the critical role of platelets during viral infection for early host survival and extended this to a human virus. The higher viremia with similar levels of type I IFN in the heart of CVB3-infected and depleted mice at 3 dpi, together with the selective enhanced expression of platelet PS, suggests that platelets may have a role in early viral dissemination independently of type I IFN by binding circulating viral particles and directing them towards macrophage phagocytosis and subsequent destruction. Moreover, the enhanced formation of platelet-neutrophil aggregates might accelerate this process. In the absence of such a mechanism, more viruses reach target organs, as noted here in the heart homogenates of infected mice, producing more severe myocarditis. Moreover, our observation of decreased levels of IgG in platelet-depleted and infected animals reveals a critical role of platelets in the later adaptive immune response.

In conclusion, our results showed that CVB interact with platelets and favored platelet-granulocyte interaction, which most likely accounts for the reduction in platelet counts. When the number of platelets, but not WBC, was reduced, survival of mice was compromised and viremia and cardiac viral burden were increased, impacting on the severity of associated disease and subsequent immune response. Future studies are necessary to further clarify the mechanisms involved and to confirm these pathogenic mechanisms in other viral diseases.

Addendum

S. Negrotto and C. J. De Giusti performed the majority of the experiments. S. Negrotto analyzed the data and wrote the paper. A. E. Ure and L. Rivadeneyra performed and analyzed the viremia studies and platelet counts. H. A. Mena performed the RT-PCR experiments. M. Schattner and R. M. Gomez designed and directed the study, and wrote and edited the paper. S. Negrotto, R. M. Gomez and M. Schattner are scientific researchers from CONICET; C. J. De Giusti, L. Rivadeneyra and A. E. Ure hold fellowships from CONICET and H. A. Mena holds a fellowship from ANPCyT.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Kinetic study of platelet depletion in C57BL/6J mice.

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Fig. S2. Absence of bleeding in mock, infected and platelet-depleted mice.

Fig. S3. Megakaryocyte (Mk) features in mock, infected and platelet-depleted mice.

Table S1. Results are expressed as means $\pm$ SEM $\times$ 10^3 uL^{-1}.

References

1 Danon D, Jerushalmy Z, De Vries A. Incorporation of influenza virus in human blood platelets in vitro. Electron microscopical observation. *Virology* 1959; **9**: 719–22.

2 Jerushalmy Z, Kohn A, De Vries A. Interaction of myxoviruses with human blood platelets in vitro. *Proc Soc Exp Biol Med* 1961; **106**: 462–6.

3 Zucker-Franklin D, Seremetis S, Zheng ZY. Internalization of human immunodeficiency virus type I and other retroviruses by megakaryocytes and platelets. *Blood* 1990; **75**: 1920–3.

4 Onlamoon N, Noisakran S, Hsiao HM, Duncan A, Villinger F, Ansari AA, Perng GC. Dengue virus-induced hemorrhage in a nonhuman primate model. *Blood* 2010; **115**: 1823–34.

5 Youssefian T, Drouin A, Masse JM, Guichard J, Cramer EM. Host defense role of platelets: engulfment of HIV and Staphylococcus aureus occurs in a specific subcellular compartment and is enhanced by platelet activation. *Blood* 2002; **99**: 4021–9.

6 Zahn A, Jennings N, Ouwehand WH, Allain JP. Hepatitis C virus interacts with human platelet glycoprotein VI. *J Gen Virol* 2006; **87**: 2243–51.

7 Alonzo MT, Lacuesta TL, Dimaano EM, Kurosu T, Suarez LA, Mapua CA, Akeda Y, Matias RR, Kuter DJ, Nagata S, Natividad FF, Oishi K. Platelet apoptosis and apoptotic platelet clearance by macrophages in secondary dengue virus infections. *J Infect Dis* 2012; **205**: 1321–9.

8 Othman M, Labelle A, Mazzetti I, Elbatarny HS, Lillicrap D. Adenovirus-induced thrombocytopenia: the role of von Willebrand factor and P-selectin in mediating accelerated platelet clearance. *Blood* 2007; **109**: 2832–9.

9 Iannacone M, Sitia G, Isogawa M, Whitmire JK, Marchese P, Chisari FV, Ruggeri ZM, Guidotti LG. Platelets prevent IFN-alpha/beta-induced lethal hemorrhage promoting CTL-dependent clearance of lymphocytic choriomeningitis virus. *Proc Natl Acad Sci U S A*. 2008; **105**: 629–34.

10 Racaniello VR. Picornaviridae: The Viruses and Their Replication. In: Fields BN, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE, Knipe DM, eds. *Virology*. Philadelphia: Lippincott Williams & Wilkins, 2001: 559–88.

11 Stanway G, Brown F, Christian P, Hovi T, Hyypiä T, King AMQ, Knowles NJ, Lemon SM, Minor PD, Pallansch MA, Palmenberg AC, Skern T. Picornaviridae. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, eds. *Virus taxonomy, VIIIth Report of the ICTV*. London, United Kingdom: Elsevier, 2004: 757–78.

12 Pallansch MA, Roos RP. Enteroviruses: Polioviruses, Coxsackieviruses, Echoviruses, and Newer Enteroviruses. In: Fields BN, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE, Knipe DM, eds. *Virology*. Philadelphia: Lippincott Williams & Wilkins, 2001: 589–630.

13 Kim KS, Hufnagel G, Chapman NM, Tracy S. The group B coxsackieviruses and myocarditis. *Rev Med Virol* 2001; **11**: 355–68.

14 Feldman AM, McNamara D. Myocarditis. *N Engl J Med* 2000; **343**: 1388–98.

15 Gupalo E, Buriachkovskaia L, Othman M. Human platelets express CAR with localization at the sites of intercellular interaction. *Virol J* 2011; **8**: 456.

16 Cihakova D, Sharma RB, Fairweather D, Afanasyeva M, Rose NR. Animal models for autoimmune myocarditis and autoimmune thyroiditis. *Methods Mol Med* 2004; **102**: 175–93.

17 Whitton JL. Immunopathology during coxsackievirus infection. *Springer Semin Immunopathol* 2002; **24**: 201–13.

18 Liu Z, Yuan J, Yanagawa B, Qiu D, McManus BM, Yang D. Coxsackievirus-induced myocarditis: new trends in treatment. *Expert Rev Anti Infect Ther* 2005; **3**: 641–50.

19 Fairweather D, Stafford KA, Sung YK. Update on coxsackievirus B3 myocarditis. *Curr Opin Rheumatol* 2012; **24**: 401–7.

20 Knowlton KU, Jeon ES, Berkley N, Wessely R, Huber S. A mutation in the puff region of VP2 attenuates the myocarditic phenotype of an infectious cDNA of the Woodruff variant of coxsackievirus B3. *J Virol* 1996; **70**: 7811–8.

21 Rinehart JE, Gomez RM, Roos RP. Molecular determinants for virulence in coxsackievirus B1 infection. *J Virol* 1997; **71**: 3986–91.

22 Cifuente JO, Ferrer MF, Jaquenod de Giusti C, Song WC, Romanowski V, Hafenstein SL, Gomez RM. Molecular determinants of disease in coxsackievirus B1 murine infection. *J Med Virol* 2011; **83**: 1571–81.

23 Romorini L, Scassa ME, Richardson GV, Bluguermann C, de Giusti CJ, Questa M, Fernandez Espinosa DD, Gomez RM, Sevlever GE, Miriuka SG. Activation of apoptotic signalling events in human embryonic stem cells upon Coxsackievirus B3 infection. *Apoptosis* 2012; **17**: 132–42.

24 Alves-Rosa F, Stanganelli C, Cabrera J, van Rooijen N, Palermo MS, Isturiz MA. Treatment with liposome-encapsulated clodronate as a new strategic approach in the management of immune thrombocytopenic purpura in a mouse model. *Blood* 2000; **96**: 2834–40.

25 Etulain J, Negrotto S, Carestia A, Pozner RG, Romaniuk MA, D'Atri LP, Klement GL, Schattner M. Acidosis downregulates platelet haemostatic functions and promotes neutrophil proinflammatory responses mediated by platelets. *Thromb Haemost* 2012; **107**: 99–110.

26 Negrotto S, De Giusti CJ, Lapponi MJ, Etulain J, Rivadeneyra L, Pozner RG, Gomez RM, Schattner M. Expression and functionality of type I interferon receptor in the megakaryocytic lineage. *J Thromb Haemost* 2011; **9**: 2477–85.

27 Etulain J, Lapponi MJ, Patrucchi SJ, Romaniuk MA, Benzadon R, Klement GL, Negrotto S, Schattner M. Hyperthermia inhibits platelet hemostatic functions and selectively regulates the release of alpha-granule proteins. *J Thromb Haemost* 2011; **9**: 1562–71.

28 Solana ME, Ferrer MF, Novoa MM, Song WC, Gomez RM. Decay-accelerating factor 1 deficiency exacerbates Trypanosoma cruzi-induced murine chronic myositis. *Muscle Nerve* 2012; **46**: 582–7.

29 Rowley JW, Schwertz H, Weyrich AS. Platelet mRNA: the meaning behind the message. *Curr Opin Hematol* 2012; **19**: 385–91.

30 Gupalo E, Kuk C, Qadura M, Buriachkovskaia L, Othman M. Platelet-adenovirus vs. inert particles interaction: effect on aggregation and the role of platelet membrane receptors. *Platelets* 2013; **24**: 383–91.

31 Ghosh K, Gangodkar S, Jain P, Shetty S, Ramjee S, Poddar P, Basu A. Imaging the interaction between dengue 2 virus and human blood platelets using atomic force and electron microscopy. *J Electron Microsc (Tokyo)* 2008; **57**: 113–8.

32 Semple JW, Italiano JE Jr, Freedman J. Platelets and the immune continuum. *Nat Rev Immunol* 2011; **11**: 264–74.

33 Shimony N, Elkin G, Kolodkin-Gal D, Krasny L, Urieli-Shoval S, Haviv YS. Analysis of adenoviral attachment to human platelets. *Virol J* 2009; **6**: 25.

34 Bergelson JM, Mohanty JG, Crowell RL, St John NF, Lublin DM, Finberg RW. Coxsackievirus B3 adapted to growth in RD cells binds to decay-accelerating factor (CD55). *J Virol* 1995; **69**: 1903–36.

35 Shafren DR, Bates RC, Agrez MV, Herd RL, Burns GF, Barry RD. Coxsackieviruses B1, B3, and B5 use decay accelerating factor as a receptor for cell attachment. *J Virol* 1995; **69**: 3873–7.

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36 Nicholson-Weller A, March JP, Rosen CE, Spicer DB, Austen KF. Surface membrane expression by human blood leukocytes and platelets of decay-accelerating factor, a regulatory protein of the complement system. *Blood* 1985; **65**: 1237–44.

37 Chung SK, Kim JY, Kim IB, Park SI, Paek KH, Nam JH. Internalization and trafficking mechanisms of coxsackievirus B3 in HeLa cells. *Virology* 2005; **333**: 31–40.

38 Forghani B, Schmidt NJ. Association of herpes simplex virus with platelets of experimentally infected mice. *Arch Virol* 1983; **76**: 269–74.

39 Bik T, Sarov I, Livne A. Interaction between vaccinia virus and human blood platelets. *Blood* 1982; **59**: 482–7.

40 Gavrilovskaya IN, Shepley M, Shaw R, Ginsberg MH, Mackow ER. beta3 Integrins mediate the cellular entry of hantaviruses that cause respiratory failure. *Proc Natl Acad Sci U S A*. 1998; **95**: 7074–9.

41 Xing L, Huhtala M, Pietiainen V, Kapyla J, Vuorinen K, Marjomaki V, Heino J, Johnson MS, Hyypia T, Cheng RH. Structural and functional analysis of integrin alpha2I domain interaction with echovirus 1. *J Biol Chem* 2004; **279**: 11632–8.

42 Koupenova M, Vitseva O, Mackay CR, Beaulieu LM, Benjamin EJ, Mick E, Kurt-Jones EA, Ravid K, Freedman JE. Platelet-TLR7 mediates host survival and platelet count during viral infection in the absence of platelet-dependent thrombosis. *Blood* 2014; **124**: 791–802.

43 Hottz ED, Oliveira MF, Nunes PC, Nogueira RM, Valls-de-Souza R, Da Poian AT, Weyrich AS, Zimmerman GA, Bozza PT, Bozza FA. Dengue induces platelet activation, mitochondrial dysfunction and cell death through mechanisms that involve DC-SIGN and caspases. *J Thromb Haemost* 2013; **11**: 951–62.

44 Loria GD, Romagnoli PA, Moseley NB, Rucavado A, Altman JD. Platelets support a protective immune response to LCMV by preventing splenic necrosis. *Blood* 2013; **121**: 940–50.

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