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Increased uptake by splenic red pulp macrophages contributes to rapid platelet turnover in WASP(–) mice

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Abstract

Thrombocytopenia due to rapid platelet consumption contributes to the severe thrombocytopenia of the Wiskott Aldrich Syndrome (WAS), and to the milder thrombocytopenia seen in murine WAS. Here we show that rapid clearance of ¹¹¹In labeled murine WASP(–) platelets correlates with enhanced splenic uptake. Using platelets labeled with a pH sensitive fluorescent marker (pHrodo), we quantify normal platelet uptake by red pulp macrophages (RPM), and demonstrate its enhancement after in vivo opsonization of platelets. The spleens of WASP(–) mice contain an increased number of RPM, and rapid clearance of WASP(–) platelets in WASP(–) mice in turn generates an increased number of pHrodo(+) splenic RPM. To separately assess the platelet intrinsic and recipient dependent functions involved in the clearance and splenic phagocyte uptake of WASP(–) platelets, we performed “crossed” pHrodo(+) platelet injection studies (WT to WASP(–), WASP(–) to WT). We show that an extrinsic effect of recipient WASP deficiency on the clearance of WASP(–) platelets correlates with increased platelet uptake by RPM. An intrinsic effect of platelet WASP deficiency on platelet clearance does not, however, correlate with increased total uptake by WT or WASP(–) RPM. In contrast to other published findings, we find no evidence of a baseline or antibody-induced increase in phosphatidyl serine exposure on WASP(–) platelets. Our findings suggest that an increased number of RPM in WASP(–) mice contributes significantly to the increased platelet consumption rate in WASP(–) mice. This may explain the consistent efficacy of splenectomy in murine and clinical WAS.

Keywords

Wiskott-Aldrich; Thrombocytopenia; phagocytosis

Introduction

The Wiskott-Aldrich Syndrome is an X-linked condition characterized in most cases by immunodeficiency, severe eczema, and thrombocytopenia[1, 2]. It is caused by mutations which reduce or eliminate expression of the WAS protein (WASP). WASP is expressed almost exclusively in blood cells, where its biochemical function involves the amplification

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of signals which begin at the cell membrane and end in actin polymerization (reviewed recently by Cleland and Siegel[3]). This function is shared by a number of cell activities, including signal transduction through the T-cell receptor, integrin-dependent adhesion of neutrophils, podosome formation by a number of cell types, and trafficking of antigen presenting cells. While those functions are all impaired by WASP deficiency, others are augmented, including BCR/TLR signal transduction in B-cells (Becker-Herman, 2011). In megakaryocytes, it is not clear whether loss of WASP activity results in impaired[4] or premature[5] proplatelet formation (or both), but the end result (from platelet kinetic studies[6–8]) is impaired platelet production in clinical WAS.

There is evidence that increased platelet consumption also contributes to the thrombocytopenia of clinical and murine WAS[9, 10]. Using a numerical analysis model, we have shown that both cis (platelet intrinsic) and trans (recipient dependent) features of WASP deficiency contribute to the rapid clearance of murine WASP(–) platelet from the circulation[11].

Splenectomy improves the platelet count in both murine[9] and clinical[12] WAS, and does so in the latter much more consistently than in other thrombocytopenias such as ITP[13]. This suggests that increased uptake by splenic macrophages could mediate the rapid clearance of WASP(–) platelets, via cis (platelet intrinsic) or trans (host) effects, or both. Such a mechanism is also supported by the increased susceptibility of antibody-opsonized murine WASP(–) platelets to ex vivo phagocytosis by bone marrow derived macrophages[9]. Platelets from WAS patients show a similar increased susceptibility to ex vivo phagocytosis by activated THP-1 cells[14, 15], although in this case the effect is evident in the absence of opsonization. While WAS patients are prone toward a number of autoimmune conditions, including (after splenectomy) episodic thrombocytopenias indistinguishable from ITP[12], a purely autoimmune etiology for their baseline thrombocytopenia seems unlikely. Indeed, when we eliminated virtually all antibodies from WASP(–) mice by crossing them onto the muMT(–/–) background, the thrombocytopenia (and rapid platelet consumption rate) was not corrected[10]. Despite that finding, an underlying proclivity toward rapid platelet clearance could render WAS patients, and WASP(–) mice, more susceptible to the effects of autoimmunity to platelet antigens.

Taken together, these studies suggest that the interaction between WASP(–) platelets and one or more types of splenic phagocyte could be critical to the rapid clearance of WASP(–) platelets from the bloodstream. Here we used two different platelet tracking methods to look more closely at how WASP(–) platelets are consumed in vivo.

Methods

Reagents

CMFDA (“celltracker green”) and pHrodo were obtained from Invitrogen. Anti-mouse CD41/PE (clone MWReg30), anti-mouse CD61 (clone 2c9G2), and Fc Block (anti-mouse CD16/CD32) were obtained from BD Biosciences. Anti-CD3/FTIC (clone 145-2C11), anti-CD19/FITC (clone 6D5), anti-NK1.1/FITC (clone PK136), anti-Ly6G/APC/Cy7 (clone 1A8), and anti-CD11b/APC (clone M1/70) were obtained from Biolegend. PGE-1 was obtained from Sigma. ¹¹¹In-oxyquinoline was obtained from GE Healthcare. 6A6 antibody, originally derived by Dr. R.A. Good[16], was prepared by standard methods from hybridoma cells provided by Dr. Jeffrey Ravetch (The Rockefeller University). Lactadherin/FITC was obtained from Haematologic Technologies Inc. Phosphate buffered saline (PBS) was obtained from Gibco. Thrombin from murine plasma was obtained from Sigma-Aldrich (catalog number T8397), resuspended in PBS at 100 units/ml, and frozen in aliquots at –80 °C.

Mouse strains

WASP(−) mice originally derived by Snapper et al.[18] were crossed onto the C57Bl/6J background for at least 8 generations. All donors and recipients in this study were 8–12 week old males. Platelet counts were routinely performed on WASP(−) mice prior to their use as platelet donors or recipients. C57Bl/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were bred and maintained in specific pathogen free environments at the Memphis VA Medical Center.

Labeled platelet preparations

Platelet preparation, labeling of platelets with CMFDA, injection of labeled platelets, and quantification of the fraction of peripheral blood platelets labeled, was performed as previously described[19]. Briefly, platelets were prepared (from blood obtained via tail clipping) using ficoll step gradients (Fico/Lyte Platelets, Atlanta Biologicals), followed by resuspension at 2.9×10^5 platelets per μl in “complete tyrode’s” (CT) buffer (20 mM Hepes, 137 mM NaCl, 13.8 mM NaHCO_3 , 2.5 mM KCl, 0.36 mM $\text{NaH}_2\text{PO}_4\text{-H}_2\text{O}$, 5.5 mM glucose, 0.25% bovine serum albumin, 1 mM MgCl_2) supplemented with 1 $\mu\text{g/ml}$ PGE-1.

Labeling with CMFDA was performed in CT/PGE-1 at 2.9×10^5 platelets per μl and 1.25 μM CMFDA, at 37 °C for 20 minutes (protected from light). After addition of five volumes CT/PGE-1, platelets were centrifuged at 2000 RCF for 10 minutes at room temperature, resuspended in CT, and counted using a Beckman-Coulter (Fullerton, CA, USA) Model Z2 particle count and size analyzer. Labeling intensity was assessed by flow cytometry, and platelet concentration was adjusted to a final concentration of 2.5×10^5 per μl . All flow cytometry was performed on a Becton Dickinson LSR cytometer configured with 355 nm, 404 nm, 488 nm, 561 nm, and 640 nm lasers, using the emission filters shown in the figure legends.

For labeling with ^{111}In -oxyquinoline, platelet preparations in CT/PGE1 at 2.9×10^5 per μl were mixed with 0.2 μCi per million platelets of ^{111}In -oxyquinoline, and incubated at 37 °C for 30 minutes with gentle agitation. After addition of 5 volumes CT/PGE-1, platelets were centrifuged at 6,000 RCF for 4.5 minutes, then resuspended in a minimum volume of CT. Platelet recovery was estimated from a parallel “mock-labeled” tube, and the volumes of labeled platelets were adjusted to a final concentration of 2.5×10^5 per μl . Specific activities of the labeled platelets ranged from 1×10^5 to 4×10^5 cpm per million platelets. 24 hours after injection of ^{111}In -labeled platelets, recipients were sacrificed and the organs shown in the figures were removed. ^{111}In cpm in total organ tissue (or representative portions quantified as a fraction of total organ weight) was then measured in a gamma counter.

Labeling with pHrodo was performed in CT/PGE-1 at 2.9×10^5 platelets per μl and 5 $\text{ng}/\mu\text{l}$ pHrodo, at 37 °C for 30 minutes (protected from light). The 1 $\mu\text{g}/\mu\text{l}$ pHrodo stock solution in DMSO used yielded a final DMSO concentration of 0.5% (v/v) in the labeling reaction. After addition of 5 volumes CT/PGE-1, platelets were centrifuged at 2000 RCF for 10 minutes at room temperature, and resuspended in CT at a final concentration of 2.0×10^5 per μl . Labeling intensity was verified by flow cytometry after dilution into PBS that had been adjusted to pH 4.0 with HCl.

In vivo platelet consumption assays

Labeled platelets or mixtures of labeled platelets were injected via tail vein. Typical injection volumes of 400 to 600 μl yielded platelet dosages of 1 to 1.5×10^8 platelets. For CMFDA+ or pHrodo+ platelets, this resulted in most cases in labeling of 2% to 4% of circulating platelets at T0 (5 minutes). Recipients were bled retro-orbitally at T0 and at the final time points described in the text.

For consumption of ^{111}In -labeled platelets, total cpm per 5 μl peripheral blood were measured with a gamma counter at both time points; decay correction was factored into the analysis of the data obtained at the second time point (the half life of ^{111}In is 2.80 days). For consumption of CMFDA+ platelets, platelets were gated using log FSC vs log SSC, gated again with the use of PE-CD41 antibody, before quantifying the fraction of CMFDA(+) platelets. For consumption of pHrodo-labeled platelets the same scheme was used, replacing PE-CD41 with FITC-CD41, and analysis was performed in PBS that had been adjusted to pH 4.0 with HCl.

Platelet counts were performed via quantitative flow cytometry by reference to an internal fluorescent particle control, as previously described[9].

Uptake of pHrodo-labeled platelets by splenocytes

Mice were sacrificed by cervical dislocation. Spleens were shredded manually and digested with collagenase D (1 mg/ml) and Dnase 1 (0.1 mg/ml) in 5 ml of HBSS at room temperature (RT) for 20 minutes. After passage through a 40 μm filter, and a second 20 minute digestion at RT, 9 volumes of red cell lysis buffer (154 mM NH_4Cl , 10 mM KHCO_3 , 1 mM disodium EDTA) and left at room temperature for two minutes. Cells were removed by low speed centrifugation (450 RCF, 10 minutes), resuspended in 1 ml PBS, underwent a second identical low speed centrifugation, and were resuspended in 1 ml PBS. Cells were counted with a Beckman-Coulter (Fullerton, CA, USA) Model Z2 particle count and size analyzer, and brought to a concentration of 1.0×10^6 per ml. After sequential binding of a viability marker (Live/Dead Blue, Invitrogen; 1 μl per 10^6 cells) and Fc block (anti CD16/CD32), cells were exposed to a cocktail of lymphocyte lineage markers (FITC-labeled anti-CD3, CD19, NK1.1) and phagocyte markers (CD11b/APC, Ly6G/APC-Cy7), or to appropriate isotype controls.

Phosphatidyl serine measurements

6A6 or anti-CD61 antibody was allowed to bind to 5×10^6 platelets (prepared as described above) in 100 μl of CT at 37 $^\circ\text{C}$ for 1 hour. 500 μl of CT was added, platelets were centrifuged at 6000 g for 5 minutes at room temperature, and the platelets were resuspended at 50,000/ μl in CT containing 120 ng/ml FITC-lactadherin. After 1 hour at room temperature platelets were analyzed by flow cytometry. Positive controls for this assay were generated by treating 10^7 WT platelets at 2×10^4 platelets per μl in PBS with 1 unit of thrombin for 10 minutes at 37 $^\circ\text{C}$. Platelets were then diluted in CT/PGE1, spun at 6K rcf for 5 minutes at room temperature, resuspended in CT, and allowed to bind FITC-lactadherin as described above. Negative controls were resuspended in CT only.

All flow cytometric data was acquired with Diva (Becton-Dickinson) software, and analyzed using Flowjo software (Tree Star Inc., Ashland, OR).

Statistical methods

p values were calculated using student's 2-tailed t-test unless otherwise noted. Data comparisons yielding a p value of less than 0.01 are labeled with double asterisks in the figures; those with p values < 0.05 but > 0.01 are labeled with single asterisks.

Results

Clearance and organ uptake of ^{111}In -labeled platelets

WASP(−) or WT mice were injected via tail vein with either WASP(−) or WT platelets labeled with ^{111}In . Total cpm in peripheral blood was measured at T0 (5 minutes after injection) and again 24 hours later. The animals were sacrificed at that time, and total organ-

specific cpm was measured in spleen, liver, lungs, kidneys, and heart. WASP(−) platelets in WASP(−) recipients were cleared rapidly from the circulation in comparison to WT platelets in WT recipients (figure 1), consistent with our previous findings using fluorescently-labeled platelets[9–11]. For WASP(−) platelets injected into WASP(−) recipients, ¹¹¹In uptake (as a fraction of total recovered cpm) was significantly greater in spleen, and significantly less in lung and heart (figure 1). These results suggest that uptake by splenic phagocytes, (and not uptake by hepatocytes or Kupffer cells) contributes significantly to the rapid clearance of WASP(−) platelets in this context. Given that the spleen is a blood-rich organ, the rapid clearance of WASP(−) platelets from the bloodstream would if anything render this result an underestimate of enhanced uptake by splenic phagocytes. We note that the significant splenomegaly seen in older WASP(−) mice[17] is minimal (mean spleen weight increase 12%, $p = 0.17$) in the 8–12 week old mice used in this study.

Splenic phagocyte populations in WASP(−) mice

To identify and quantify splenic phagocytes which could mediate the increased clearance of WASP(−) platelets, we used both immunophenotype and autofluorescence (figure 2) as described by Mitchell et al. [18]. To accommodate detection of fluorescence transferred from labeled platelets (see below), we truncated the immunophenotyping method to detection of (autofluorescent) red pulp macrophages (RPM), neutrophils, and two mixed populations of phagocytes we termed pX and pY. pX (CD11b−, Ly6G−) is an uncharacterized population, while pY (CD11b+, Ly6G−) includes monocytes, eosinophils, and dendritic cells[18]. We found that the spleens of WASP(−) mice contain a significantly higher fraction of each of these phagocyte populations than do spleens from WT mice (figure 3). Net leukocyte recovery per the (slightly larger) WASP(−) spleens trended higher as well (4.67×10^7 per spleen, vs. 3.82×10^7 per WT spleen; $p = 0.22$, student's t-test).

Clearance and splenic phagocyte uptake of pHrodo labeled platelets

We then developed a means of tracking the uptake of fluorescently labeled WT and WASP(−) platelets in these cell types. Fluorescent probes we have used to label platelets in the past (CMFDA, DIO) are poorly suited for tracking uptake by phagocytes, in part because their emission spectra overlap with the autofluorescence used to identify the most phagocytically active cells in the spleen (RPM). The pH sensitive fluorescent marker, PHrodo (Invitrogen), with longer wavelength excitation and emission spectra, both mitigates this problem and enhances the sensitivity of uptake detection in low-pH intracellular environments.

To determine whether labeling of platelets with PHrodo artifactually alters platelet consumption, we compared the in vivo clearance rates of CMFDA and pHrodo labeled platelets (WT and WASP(−) in the context of either recipient genotype. We saw no significant difference in the clearance rate of the two types of labeled platelets, although there is a trend toward more rapid clearance of pHrodo+ platelets (figure 4).

We then assessed the uptake of platelet-derived pHrodo in splenic phagocyte populations as gated in figure 3. Because we expected quenching to reduce the sensitivity of the study over longer time periods, we evaluated uptake at 6 hours post injection. We found that after injection of WT platelets, a high percentage of RPM demonstrated uptake, while lower uptake rates could be detected in the other populations (figure 5). Our concern about quenching was born out by our finding that the fraction of RPM showing PHrodo uptake at 24 hours after injection was 10–20% lower than the fraction seen at 6 hours (data not shown).

To verify the biological relevance of RPM uptake of pHrodo(+) platelets, we evaluated the clearance and splenic phagocyte uptake of WT pHrodo(+) platelets after their clearance was accelerated (1 hour after platelet injection) by injection of a clearance-inducing antibody (6A6)[16]. Representative findings three hours after antibody injection are shown in figure 6. As expected, 6A6 induced both a marked thrombocytopenia and an approximate doubling of the clearance of the labeled platelets (figure 6a, from ~45% cleared to ~90% cleared). This correlated with a roughly two-fold increase in the fraction of RPM showing PHrodo uptake (figure 6B).

Effects of WASP deficiency on uptake of pHrodo labeled platelets by RPM

The clearance rates seen with pHrodo(+) WASP(–)-to-WASP(–) vs WT-to-WT studies at 6 hr post-injection (figure 7), paralleled those seen at 24 hr with ¹¹¹In-labeled platelets (figure 1). The increased clearance of WASP(–) platelets in WASP(–) recipients correlated with an over two-fold increase in the number of pHrodo(+) RPM. This is likely to be an underestimate, given the above-noted trend toward a higher cell count in the spleens of WASP(–) mice in this age range. The *fraction* of PHrodo(+) RPM was not significantly different, suggesting that the increased baseline number of RPM in WASP(–) mice contributes to the increased clearance of WASP(–) platelets.

To further test that conclusion, we performed “crossed” studies of platelet clearance (WT into WASP(–), WASP(–) into WT). These allow separate consideration of *extrinsic* (recipient, or *trans*) and *intrinsic* (donor, or *cis*) effects of WASP deficiency on platelet clearance and on RPM uptake. Our results (figure 8) suggest that both platelet extrinsic and platelet intrinsic effects contribute to enhanced clearance of WASP(–) platelets, consistent with our previous studies[11]. Specifically, the *extrinsic* effect on clearance of WASP(–) platelets correlates with generation of an increased number of pHrodo(+) RPM (figure 8C). The *platelet intrinsic* effect of WASP deficiency on platelet clearance, however, does not correlate with increased total clearance by RPM in WASP(–) recipients (figure 8C, compare (i) wasp-/wasp- to wt/wasp-, and (ii) wasp-/wt to wt/wt), although a significant platelet intrinsic effect on the fraction of pHrodo(+) RPM is evident in WT recipients (figure 8B) (see discussion). The data does not consistently favor a requirement for both intrinsic and extrinsic WASP deficiency (i.e. a “cis/trans” mechanism) for either platelet clearance or uptake by RPM.

Phosphatidyl serine exposure on WASP(–) platelets

To investigate a potential contributor to the platelet intrinsic effect of WASP deficiency on clearance rate, we quantified platelet surface phosphatidyl serine exposure using the fluorescent marker lactadherin. In contrast to a published report[19], we found no baseline increase in PS exposure (figure 9). We also were unable to induce increased PS exposure with 37 degree incubation, or with exposure to 6A6 antibody.

Discussion

WASP(–) mice demonstrate a thrombocytopenia which is in part due to rapid platelet clearance from the circulation. Here we show that this correlates with increased splenic clearance of ¹¹¹In-labeled WASP(–) platelets in WASP(–) recipients. To trace that clearance to specific splenic phagocytes, we labeled platelets with the pH-sensitive fluorescent marker pHrodo. We show that this does not introduce artifactual changes in platelet clearance rate, and that it can be used to track the *in vivo* clearance of antibody-opsonized platelets by red pulp macrophages (RPM). This method improves significantly on previous means of tracking the phagocytosis of platelets by (A) avoiding use of non-specific macrophage markers such as F4/80[18]; (B) enhancing detection of internalized platelets in

a low pH intracellular environment; and (C) avoidance of fluorescent emission overlap between the (autofluorescent) RPM and the marker used to track uptake.

We've shown that the clearance of pHrodo(+) WASP(−) platelets is mediated largely by red pulp macrophages (RPM). We then used “crossed” pHrodo(+) platelet turnover studies (WASP(−)-to-WT, WT-to-WASP(−)) designed to separately study how *cis* (platelet intrinsic) and *trans* (recipient dependent) effects contribute to the increased clearance of WASP(−) platelets. Interpretation of this type of study is complicated, however, by the secondary effects of thrombocytopenia itself (regardless of its etiology) on (A) the donor platelet age distribution, and (B) the recipient's platelet turnover rate[20]. Using a numerical analysis method to separately model platelet age distributions as well as random and lifespan-dependent consumption rates, we have quantified these secondary effects, and confirmed substantial platelet intrinsic (*cis*) and platelet extrinsic (*trans*) effects of WASP deficiency on the rate of random destruction of platelets labeled with CMFDA or BMQC[11]. Here we show that these effects are also evident in the net clearance rate of pHrodo-labeled WASP(−) platelets (figure 8).

We then asked whether these effects are evident in the rate of uptake of pHrodo(+) WASP(−) platelets by splenic phagocytes. The *extrinsic* effect of (recipient) WASP deficiency on pHrodo(+) platelet clearance correlates with an increased number of pHrodo(+) RPM in the spleens of WASP(−) mice. This appears to be a consequence of a “baseline” increase in the number of splenic RPM in WASP(−) mice. Increased uptake of platelets by CD68+ splenic macrophages in WAS patients[21] suggests that the same process pertains in clinical WAS. Such an extrinsic effect may explain why the thrombocytopenia of WAS responds more consistently to splenectomy than is the case for other thrombocytopenias such as ITP.

Increased splenic RPM could in turn result from impaired migration of WASP(−) macrophages *out* of the spleen. This would be expected by analogy to the effects of WASP deficiency on the migration of other cell types and, in particular, its inhibitory effect on podosome turnover[22].

The *intrinsic* effect of (platelet) WASP deficiency on platelet clearance does not correlate with increased total uptake by WASP(−) RPM, or (in our hands) with increased phosphatidyl serine exposure. Given that we saw no increased uptake of ¹¹¹In-labeled WASP(−) platelets by the other tissues we surveyed (figure 1), this suggests that murine WASP(−) platelets could be more susceptible than WT platelets to mechanical or complement-mediated intravascular lysis.

Failure to detect an intrinsic effect of platelet WASP deficiency on total uptake of platelets by RPM could correlate with our inability to detect increased *ex vivo* uptake of murine WASP(−) platelets by bone marrow derived macrophages (although such an effect of WASP deficiency is evident after antibody opsonization)[9]. Human WASP(−) platelets, in contrast, show increased *ex vivo* uptake by a macrophage cell line (THP-1 cells) even without opsonization [14, 15]. These contrasting findings suggests that an intrinsic effect of human, but not murine, platelet WASP deficiency on uptake by splenic macrophages could contribute to the more severe thrombocytopenia seen in clinical vs. murine WAS.

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A) ^{111}In -Platelet in circulation at 24 hr B) ^{111}In -cpm in organs **Figure 1.**

Clearance and in vivo organ uptake of ^{111}In Indium-labeled platelets 24 hours after platelet injection. The data is pooled from two separate experiments. A) ^{111}In - Platelets cleared at 24 hr. Total peripheral blood cpm was measured at time zero (five minutes after injection) and at 24 hours. The latter counts were corrected for the expected amount of radioactive decay. $n=7$ (WASP(-) to WASP(-); $n=8$ (WT to WT). B) ^{111}In -cpm in organs. Recovered cpm per organ are shown as of total counts recovered in the five organs shown. $n=13$ each. Double asterisks: $p < 0.01$.

Figure 2.

Identification of splenocyte populations. The data shown is from a WT mouse injected with pHrodo(+) WT platelets. Intact, non-dimeric, live cells were distinguished from lymphocyte lineages with the FITC markers CD3, CD19, and NK 1.1. Red pulp macrophages were identified by their autofluorescence. Neutrophils were identified in lineage negative cells as CD11b⁺, Ly6G⁺ cells. The populations labeled pX and pY are discussed in the text. Trace levels of an artifactual CD11b⁻ population (such as that seen at the left in the last plot) were seen in some studies.

Figure 3.

Splenic phagocytes in WT and WASP(-) mice. Splenocytes were gated as shown in figure 2. The data is pooled from 18 WT mice (4 untreated, 14 used as recipients of pHrodo(+) platelets) and 22 WASP(-) mice (4 untreated, 18 recipients of pHrodo(+) platelets) obtained in the course of six separate experiments. All of the differences shown are significant to $p < 0.01$. Injection of pHrodo(+) platelets had no significant effect on cell counts. There is a non-significant trend toward a higher yield of total splenic leukocytes from the WASP(-) mice in this study ($4.67E7$ vs $3.82E7$, $p = 0.22$ student's t-test).

Figure 4.

16 hr clearance of Phrodo(+) vs CMFDA(+) platelets. An equal number of (CMFDA+) and (pHrodo+) platelets were mixed (total $10E8$ platelets) and injected into recipients. The fraction of labeled circulating platelets for each population was measured at T0 (5 minutes after injection) and again at 16 hours post-injection. Data shown is the T(16) fraction as a percentage of T(0) for each of 11 recipients. Each pair of columns shows the data obtained in a single recipient. Donor and recipient genotypes are shown. The mean ratio of phrodo(+) to CMFDA(+) platelets for the 11 recipients shown is 0.90 (SD 0.24).

Figure 5.

Phrodo(+) platelet uptake by WT splenic phagocytes. Splenocytes were prepared 6 hours after injection of pHrodo(+) WT platelets into WT recipients. Populations were resolved as shown in figure 2. Contour plot resolution for red pulp macrophages is at 5% per line (with individual outliers shown). All other contour plots show a higher (2%) resolution.

A) Clearance of total and Phrodo(+) platelets

B) Uptake of Phrodo by splenic phagocytes

Figure 6.

Antibody-induced platelet clearance and uptake by RPM. pHrodo(+) WT platelets were injected into four WT recipients. Platelet counts, and the fraction of peripheral blood platelets labeled with pHrodo, were measured 5 minutes later (T0). One hour after platelet injection, two recipients were injected with approximately 3 μ g each of 6A6 antibody. The other two recipients were injected with an equal volume of saline. A) Three hours after platelet injection, peripheral blood platelet counts, and the pHrodo(+) fraction of peripheral blood platelets, were measured. B) Four hours after platelet injection, the recipients were sacrificed and the pHrodo(+) fraction of splenic phagocytes (resolved as in figure 2) was determined.

A) Phrodo(+) platelets in circulation at 6 hr

B) Splenic phagocyte uptake of pHrodo(+) platelets

Figure 7.

Platelet clearance and splenic phagocyte uptake in WT and WASP(-) mice. A) Clearance of pHrodo(+) platelets. Data is the fraction of pHrodo(+) platelets at T(6) normalized to the same fraction at T(0). n=5 (WT to WT); n=8 (WASP(-) to WASP(-)). B) Splenic phagocyte uptake of pHrodo(+) platelets. Phrodo(+) and Phrodo(-) splenocytes the mice in (A) were gated as shown in figures 2 and 5. Double asterisks: $p < 0.01$

Figure 8.

Intrinsic and extrinsic effects on platelet clearance and uptake by RPM. pHrodo(+) platelets were injected via tail vein. The fraction of peripheral blood platelets labeled with pHrodo was evaluated at 5 minutes after injection, and again at 6 hours. At the latter time, recipients were sacrificed and uptake of pHrodo by splenic red pulp macrophages (RPM) was measured. Donor/recipient pairings are shown. WASP(-)-to- WASP(-) mice, n=8; WASP(-) to WT mice, n=9; WT to WASP(-) mice, n=10; WT to WT mice, n=5. A) Effects on pHrodo(+) platelet clearance. B) Effects on platelet uptake by RPM (% pHrodo(+)). C) Effects on platelet uptake by RPM (pHrodo(+) RPM, % of splenocytes). Significant differences are shown (*, $p < 0.05$; **, $p < 0.01$, student's t-test, two-tailed).

A) Gating strategy **B) Thrombin treated controls** **C) Effect of 37 degree incubation** **D) Anti-CD61** **E) 6A6** **Figure 9.**

Phosphatidyl serine exposure in response to antibody binding. A) Gating strategy. A well defined FSC-low, FITC-lactadherin(+) platelet population was in most cases evident, and became more distinct after binding of anti-CD61 antibody. The data shown is from WASP(-) platelets treated with 450 ng per million platelets of anti-CD61 as described in materials and methods. B) Thrombin treated controls. WT platelets were treated with thrombin as described in materials and methods, then exposed to lactadherin and gated as in (A). C) Effect of 37 degree incubation. Platelets were left at RT or at 37 degrees for 1 hour, then analyzed as in (A). D,E) Platelets were exposed to the indicated amounts of antibody at 37 degrees for 1 hour (diamonds) or three hours (squares). The “0” antibody specimens were exposed to an isotype control antibody at 150 ng (C) or 15 ng (D) per million platelets. The differences shown are not significant ($p > 0.05$, student's t-test). For panels C and D, results were pooled from 8 separate experiments (7 performed in triplicate, one in duplicate). For panel E, results are pooled from 3 separate experiments performed in triplicate. Error bars are standard errors.