CME/SAM

Platelets made HLA deficient by acid treatment aggregate normally and escape destruction by complement and phagocytes in the presence of HLA antibodies

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BACKGROUND: The presence of antibodies against HLA Class I can lead to platelet (PLT) transfusion refractoriness, that is, the repeated failure to achieve adequate posttransfusion PLT count increments. PLT refractoriness can be overcome by transfusion of HLA-matched donor PLTs. A different approach is to remove HLA from the PLT surface using low pH. Previous case studies using HLA-stripped PLTs showed encouraging but inconsistent results and lacked information on the biologic effects of acid treatment on PLT function as well as sensitivity to PLT destruction in the presence of HLA antibodies.

STUDY DESIGN AND METHODS: PLTs prepared from buffy coats were stripped from HLA Class I using a brief incubation at pH 2.9. Kinetics of acid stripping, viability, phenotypic alterations, and sensitivity to complement-mediated lysis and phagocytosis were determined by flow cytometry. Functional potential was evaluated using a multiplate analyzer.

RESULTS: Acid-treated PLTs were viable, upregulated activation markers normally and aggregated to a similar extent as untreated PLTs in response to stimulation with three natural agonists. Acid treatment removed 70% to 90% of HLA Class I complexes from the PLT surface, which led to complete protection from HLA antibody–mediated complement lysis and reduced monocyte-mediated phagocytosis in the presence of anti-HLA in vitro.

CONCLUSION: Our study fills an important knowledge gap in how acid treatment affects PLT function and interactions with immune cells, paving the way for controlled clinical trials to evaluate acid-treated PLTs as an alternative to HLA-matched donors in PLT refractoriness.

Thrombocytopenia is frequently found in patients with hematologic malignancies.[1] The standard therapy to stop or prevent bleeding in these patients is transfusion of platelet (PLT) concentrates.[1,2] If PLT transfusions fail to increase PLT count, patients are considered refractory. Refractoriness can depend on infection, fever, or ongoing bleeding, but can also be caused by antibodies against nonself HLA Class I molecules.[2-6] Repeated transfusions are a major risk factor for HLA immunizations. Reducing white blood cells in blood products lowers the incidences of both HLA immunizations and PLT refractoriness.[7] However, 3% of patients still develop refractoriness to subsequent PLT

ABBREVIATIONS: BC(s) = buffy coat(s); FSC = forward scatter; MMP = mitochondrial membrane potential; RT = room temperature; SSC = side scatter; TRAP-6 = thrombin receptor–activating peptide 6; WB = whole blood.

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transfusions,[7-9] showing that PLT refractoriness caused by antibodies to HLA Class I remains a clinical problem.

When PLTs are transfused to refractory patients with antibodies against HLA Class I they are removed from the circulation by complement[10] or by Fc receptor–expressing phagocytes.[11] The current approach to overcome refractoriness caused by anti-HLA antibodies is transfusion of HLA-matched PLTs.[2] The logistics required to obtain HLA-matched PLTs for each patient are complicated, especially in cases of emergency, and not all patients with anti-HLA antibodies are treated optimally.[2]

A different approach to treat PLT refractoriness is to remove the antigenic structure of donor HLA Class I from PLTs before transfusion.[12,13] A short treatment with citric acid leads to denaturation of the trimolecular HLA complexes without significant damage to the PLTs.[13] There are seven case reports of treatment of refractory patients with such acid-treated PLTs.[14-17] In four, bleeding stopped or small PLT increments were obtained,[14,16,17] but in the other three, the transfusions did not result in a sufficient PLT increment,[15,16] and in one of the patients a febrile transfusion reaction was reported.[16]

The variable outcomes of the case reports and the limited information regarding function of acid-treated PLTs and their interactions with immune effector mechanisms makes it difficult to evaluate acid-treated PLTs as an alternative transfusion strategy to HLA-immunized individuals. Studies so far suggest that most PLTs survive the acid treatment and maintain some function and that reactivity to anti-HLA Class I antibodies decreases.[13,16,18-20] No study has tested whether acid treatment protects PLTs from complement activation and phagocytosis. Furthermore, it has not been clarified whether the poor effect of acid-treated PLTs in some studies resulted from toxic effects on the PLTs, as studies investigating PLT function after exposure to low pH came to contradictory results.[13,18]

Because of the great clinical and logistic benefits HLA-stripped PLTs would represent, we decided to investigate these questions in more detail. First, we determined the kinetics of HLA removal, which is important to identify the optimal protocol for HLA stripping in a clinical setting. Second, we show that acid-treated PLTs were protected from both HLA antibody–mediated complement activation and from monocyte-mediated phagocytosis. Finally, an extensive functional characterization showed no reduction in the ability of acid-treated platelets to aggregate after stimulation with three agonists that regulate PLT function in vivo. Our data suggest that acid-treated PLTs have the potential to survive with intact functional capacity in PLT refractory patients with anti-HLA antibodies and that the reduction in HLA expression will prevent antibody-dependent immune destruction.

MATERIALS AND METHODS

Preparation of PLT concentrates

PLTs were made from buffy coats (BCs) from regular blood donors. A total of 450 mL of whole blood (WB) was drawn into a CPD/SAG-M quadruple-bag blood container system (Fenwal, La Châtre, France) or a blood bag system (NPT 6280LE, MacoPharma, Mouvaux, France). After storage at room temperature (RT) for 2–6 hours, WB units were centrifuged (2700 $\times$ *g*) for 10 minutes at 22°C. Automatic equipment was used for preparation of blood components (Optipress, Fenwal or Macopress Smart, MacoPharma), including the BC. All BC units were mixed in pools of 5 (BC pool volume range, 216 $\pm$ 3 mL) and PLTs were prepared on Day 1 as described previously.[21]

Human samples

EDTA plasma was obtained from frozen samples from PLT-refractory patients or from mothers that had been HLA immunized during pregnancy. No specific sampling was requested from either patients or healthy volunteers for this study.

Ethical considerations

The use of normal PLTs and plasma samples containing HLA antibodies were approved by an ethical committee.

Acid treatment of PLTs

The protocol for acid treatment was modified from that of Novotny and colleagues:[16] PLTs from concentrates were pelleted, cooled on ice, and resuspended at 1×10^9 to 3×10^9 PLTs/mL in ice-cold citric acid buffer (equal volumes of 263 mmol/L citric acid and 123 mmol/L Na_2HPO_4, resulting in pH 2.9 to 3.0) or ice-cold storage solution for PLTs (SSP+, MacoPharma; control). Treatment was stopped by adding a 20-fold excess volume of ice-cold SSP+. PLTs were pelleted and resuspended in their original supernatant and counted using a cell counter (CASY TT, Roche Diagnostics, Rotkreuz, Switzerland; 60-µm capillary). Untreated samples were adjusted to match the concentrations of the treated samples.

Analysis of mitochondrial membrane potential

Loss of mitochondrial membrane potential (MMP) is an indicator of proapoptotic events in damaged PLTs and can be investigated using the dye JC-1,[22] a green fluorescent dye that forms red fluorescent aggregates inside intact mitochondria. The MMP was measured using a mitochondrial permeability transition detection kit (MitoPT JC-1, ImmunoChemistry Technologies, LCC, Bloomington, MN) as described.[23] In brief, samples were stained with JC-1 at 37°C for 15 minutes and analyzed using flow cytometry (FC500, Beckman Coulter, Marseille, France). Depolarized mitochondria (positive control) were

prepared by incubating PLTs with 5 μmol/L carbonylcyanide *m*-chlorophenylhydrazone for 30 minutes at 37°C.

Flow cytometry

The following antibodies were used: BD Bioscience (San Jose, CA)—anti-CD14 (MΦP9) allophycocyanin (APC)-Cy7, anti-CD41a (HIP8) APC, anti-CD62P (AK-4) phycoerythrin (PE)-Cy5, anti-CD63 (H5C6) PE, anti-ICAM-2 (CBR-1C2/2.2) PE, anti-NK1.1 (PK136) fluorescein isothiocyanate (FITC; isotype control for anti-HLA-A,B,C FITC), PAC-1 FITC; BioLegend (San Diego, CA)—anti-β_2-microglobulin (2M2) APC, anti-CD29 (TS2/16) APC, anti-CD49b (AK-7) FITC, anti-HLA-A,B,C (W6/32) FITC; Immunotech Beckman Coulter (Marseille, France)—anti-CD61 (SZ21) FITC, anti-human immunoglobulin G (IgG) (H2) PE; Dako Denmark (Glostrup, Denmark)—anti-C1q FITC, anti-C3c FITC; and eBioscience (San Diego, CA)—anti-CD42a (GR-P) eFluor450, anti-HLA-A,B,C free heavy chain (A4) APC.

Peripheral blood mononuclear cells (PBMNCs) were stained with fixable aqua dead cell stain (LIVE/DEAD, Molecular Probes, Paisley, UK) before incubation with antibodies. Unless stated otherwise flow cytometry was performed using a cell analyzer (BD LSRFortessa, BD Biosciences). Data were analyzed with computer software (FlowJo, Treestar, Ashland, OR). For the calculation of relative HLA expression levels the mean fluorescence intensity (MFI) of samples incubated with the isotype control was subtracted from the MFI of PLTs stained with anti-HLA-A,B,C and the corrected MFIs of treated samples were divided by the MFI of the corresponding untreated sample.

PLT aggregometry

PLT aggregation was measured using a multiplate analyzer (Roche Diagnostics). PLT concentrates were diluted fourfold with phosphate-buffered saline (PBS) containing 1.33 mmol/L $CaCl_2$, before stimulation with thrombin receptor–activating peptide 6 (TRAP-6) or collagen. Before stimulation with arachidonic acid, PLTs were diluted with the filtered supernatant of BCs obtained by density gradient centrifugation of WB using density gradient medium (Lymphoprep, Fresenius Kabi Norge, Oslo, Norway) supplemented with 1.33 mmol/L $CaCl_2$. Aggregation was measured for 8 minutes after addition of TRAP-6 (32.3 μmol/L), collagen (3.2 μg/mL), or arachidonic acid (0.5 mmol/L; all from Roche Diagnostics, Mannheim, Germany).

PLT immunofluorescence test

Anti-HLA binding from patient plasma was detected with PLT immunofluorescence test as described elsewhere,[24] using random-donor PLTs and anti-human IgG PE as secondary antibody.

Phagocytosis assay

PLTs (1.5×10^9/mL) were labeled with 51 nmol/L succinimidyl ester (pHrodo Red, Molecular Probes) in PBS for 5 minutes at RT, pelleted, and subsequently resuspended in SSP+ or used for acid treatment. PLTs were then sensitized with 10 μg/mL mouse anti-human HLA-A,B,C (W6/32) for 30 minutes at RT, washed, and resuspended in assay medium (RPMI 1640 + glutamine [Gibco, Grand Island, NY] with 10% fetal bovine serum [FBS; Gibco]).

PBMNCs isolated from BC were cryopreserved in FBS with 10% dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO). Before each assay, PBMNCs were thawed and resuspended in assay medium. PBMNCs (1×10^6 in 100 μL) and PLTs (50×10^6 in 20 to 50 μL) were incubated at 37°C in a 96-well plate (Costar Ultra-low Cluster, Corning, NY). In negative controls, phagocytosis was inhibited by keeping the samples on ice all the time or by blocking actin polymerization with 5 μg/mL cytochalasin D (Sigma-Aldrich) from 30 minutes before PLT addition. Samples were stained for flow cytometry after 2 hours. Monocytes were identified by forward scatter/side scatter (FSC/SSC) and CD14 expression.

Complement activation assay

PLTs (1.5×10^9/mL) were incubated with 2.5 μg/mL calcein red-orange AM (Molecular Probes) in the original storage solution (SSP+, 30% plasma) for 10 minutes at 37°C, washed, and resuspended in PBS or directly used for acid treatment. PLT concentrations in the samples were adjusted to similar counts, and 10 μL of each PLT suspension was added to 50 μL of PBS, PBS mixed 1:1 with human plasma, with or without 5 μg/mL mouse anti-human HLA-A,B,C antibody, or anti-HLA serum mixed 1:1 with human plasma in a 96-well plate (Costar Ultra-low Cluster, Corning) and incubated for 10 minutes at 37°C. Twenty microliters of each sample was stained for CD42a and C1q or C3c immediately and analyzed by flow cytometry.

Statistical analysis

Statistical analysis was performed with computer software (Prism 6.0, GraphPad Software, Inc., San Diego, CA). Unless stated otherwise the statistical significance of differences between the results for untreated, control-treated, and acid-treated PLTs was determined using one-way analysis of variance (ANOVA) with $\alpha = 5\%$ followed by Tukey's multiple comparisons test.

RESULTS

Kinetics of HLA Class I dissociation at low pH

The effect of low pH on the expression level of HLA Class I was tested using monoclonal antibodies (MoAbs) to the HLA-A, B and C in their native conformation (W6/32), to

β_2-microgloblin (2M2) or to an epitope on denatured free HLA heavy chains (A4). Acid-treatment led to loss of β_2-microgloblin from the PLT surface along with the conformation of the HLA Class I complex. Staining with antibody A4 showed that heavy chains remained at the cell surface but had lost their native conformation (Fig. 1A). Denaturation of the complexes was rapid, resulting in loss of the native conformation already after 30 seconds at low pH (Fig. 1B). Results from seven independent experiments revealed high variability of HLA reduction during the first 2 minutes of treatment, but after 5 minutes the remaining native heavy-chain signal was consistently reduced to approximately 10% to 20% (Fig. 1C). Longer treatment did not lead to further reduction, and a 5-minute treatment period was used in subsequent experiments.

Effects of acid treatment on PLT viability

The mean PLT recovery after 5 minutes of acid treatment was 73% and after 10 minutes 64% (Fig. 2A). Control-treated samples showed a similar recovery indicating that the loss of PLTs was most likely not due to acid-induced damage but to the handling. A small increase in the number of events with a diameter of more than 4 μm was seen in acid-treated PLTs (Supplementary Fig. 1, available as supporting information in the online version of this paper), suggesting that some PLTs may have aggregated. A similar observation was made when comparing FSC/SSC properties of the PLTs in flow cytometry (Fig. 2B). The presence of a "tail" of events with higher FSC and SSC values in acid-treated samples suggested an increased size and/or change of shape in some of the PLTs. Acid-treated PLTs did not show any increased loss of MMP compared to untreated reference samples (Fig. 2C), indicating that the treatment did not have an immediate effect on PLT viability.

PLT function is intact after acid treatment

Next, the influence of acid treatment on other cell surface molecules than HLA Class I was investigated. There was no statistically significant change in the expression levels of the fibrinogen receptor (CD41/CD61), ICAM-2, one component of the von Willebrand factor receptor (CD42a), and the collagen receptor integrin $\alpha_2\beta_1$ (CD49b/CD29), suggesting that sensitivity to low pH is a specific property of the HLA Class I complex (Fig. 3A). To test if acid treatment activated the PLTs, the expression level of three activation markers (PAC-1, CD62P, and CD63) and their upregulation were measured in response to stimulation. All markers were strongly upregulated on both control and acid-treated PLTs after stimulation with TRAP-6 (Fig. 3B).

There was a small basal increase in activation on unstimulated acid-treated PLTs compared to untreated PLTs (Fig. 3C). This activation was caused by the exposure to low pH and not by mechanic stimulation during centrifugation or pipetting, as control-treated PLTs showed no signs of activation. However, despite this, acid-treated PLTs clearly upregulated all activation markers to similar levels as control and untreated PLTs after stimulation (Fig. 3C), indicating that the treatment overall did not impair activation of PLTs. Finally, the ability of acid-treated PLTs to aggregate in response to different stimuli was tested using a multiplate analyzer. First it was confirmed that normal PLTs from concentrates could aggregate after stimulation with collagen, TRAP-6, and arachidonic acid (Fig. 4A). Next, acid-treated PLTs were tested in the same settings and were found to respond equally well (Fig. 4B). In response to collagen, acid-treated PLTs appeared to aggregate more efficiently compared to control PLTs (Fig. 4B).

Acid treatment prevents binding of anti-HLA from immunized patients

The PLT immunofluorescence test was applied to investigate if acid treatment reduced binding of anti-HLA from immunized patients. The HLA genotype of the PLT donors in these experiments was unknown, suggesting that in cases where no IgG were found on untreated PLTs, the specificities of the antibodies likely did not match the HLA of the random donors (Fig. 5). However, when patient antibodies did react to the test PLTs, acid treatment completely prevented binding in all cases (Fig. 5). Thus, a reduction in PLT HLA Class I by approximately 80% was sufficient to prevent significant binding of patient anti-HLA. In addition, the results also suggested that patients' HLA Class I antibodies did not react with epitopes on denatured HLA free heavy chains, at least not for the tested samples.

Acid treatment reduces HLA antibody–mediated phagocytosis

Fc receptor-mediated phagocytosis is considered to be an important pathway for the removal of antibody-coated PLTs from the circulation in immunized patients.[11,25] One difficulty with flow cytometry-based assays to measure phagocytosis is that PLTs adhere easily to monocytes even in the absence of PLT antibodies.[25] To circumvent this problem, we made use of the pH-sensitive dye pHrodo, which has a low fluorescence at neutral pH that increases with decreasing pH, for example, in acidified phagolysosomes,[26] thus allowing distinction between cell surface attachment of PLTs and active phagocytosis (Supplementary Fig. 2, available as supporting information in the online version of this paper). Only PLTs coated with antibody were phagocytosed, as seen by the appearance of a monocyte population with brighter pHrodo fluorescence (Fig. 6A). A role for phagocytosis in this process was strengthened by the finding that no increase of the pHrodo signal was detected in samples that had been

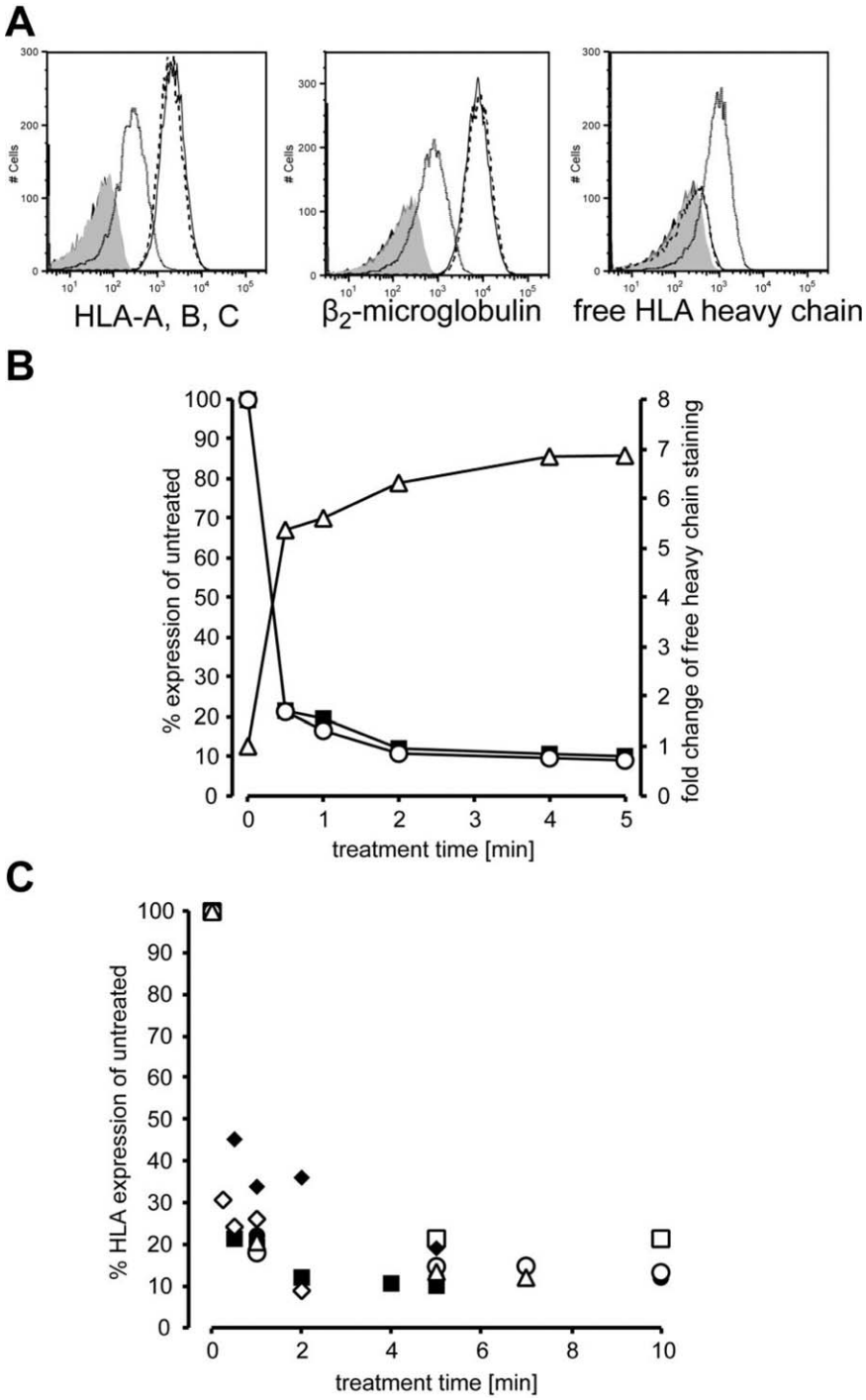


Fig. 1. Kinetics of HLA Class I complex dissociation at low pH. (A) Representative histograms for surface staining of HLA-A, -B, and -C Class I complexes; β_2-microglobulin; and HLA free heavy chain on untreated (black line), 5 minutes control-treated (dashed line), and 5 minutes acid-treated (dotted line) PLTs. Gray histograms represent isotype controls. (B) The surface staining was quantified on PLTs treated with acid for the indicated times. Data shown are from one representative out of three independent experiments. (■) HLA-ABC native; (△) HLA-ABC free heavy chain; (○) β_2-microglobulin. (C) Comparison of the time-dependent extent of HLA Class I denaturation in seven independent experiments. The different experiments are represented by different symbols.

incubated on ice or in the presence of cytochalasin D (Fig. 6A). When acid-treated PLTs were used, the percentage of pHrodo-bright monocytes was strongly reduced compared to control samples (Figs. 6B and 6C), suggesting that acid treatment has the potential to protect PLTs from phagocytosis in patients with anti-HLA. We also conclude

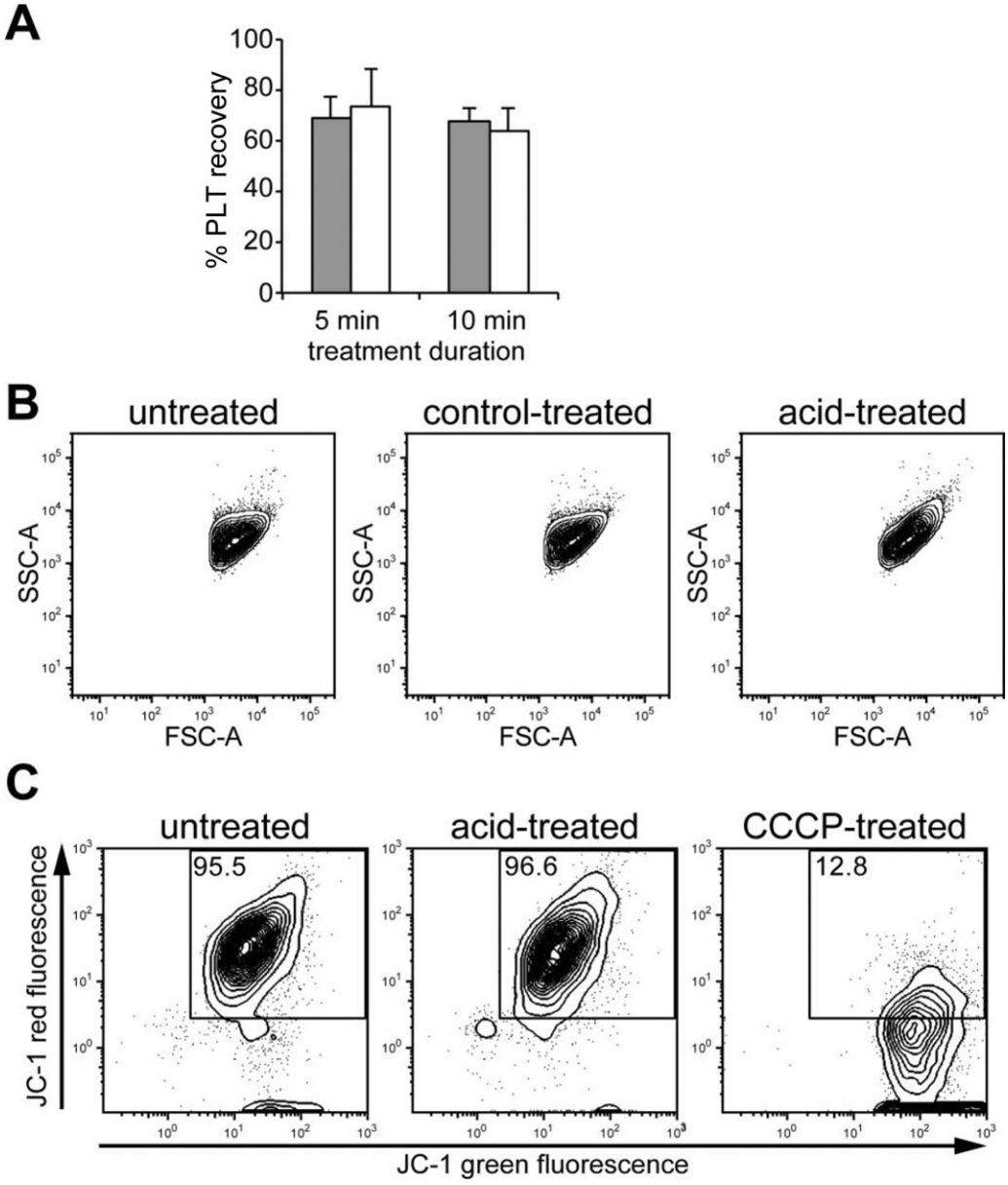


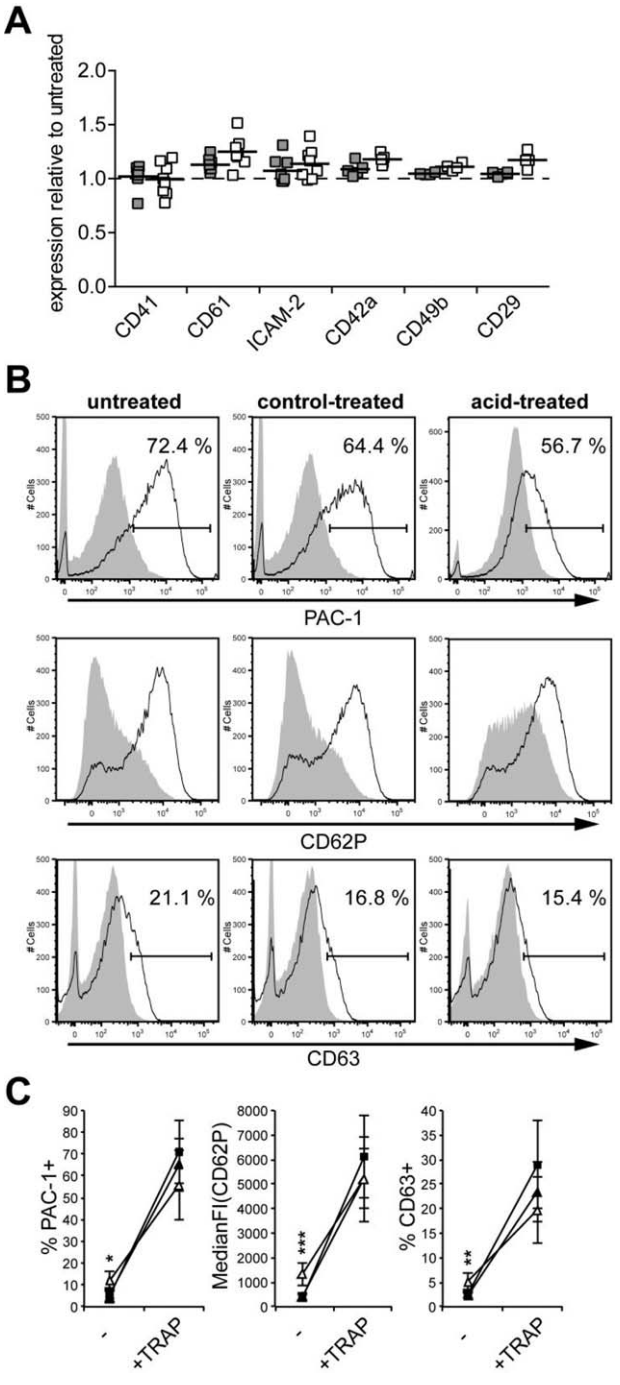
Fig. 2. PLTs are viable after acid treatment. (A) PLTs were treated for 5 minutes (n = 9) or 10 minutes (n = 5) and recovery was calculated. Plotted are means ± SD. (▓) Control-treated; (□) acid-treated. (B) Representative FSC/SSC plots of untreated, control-treated, and acid-treated PLTs. (C) PLT viability was analyzed by measurement of the MMP using the dye JC-1. Representative plots of eight samples are shown. PLTs incubated with the mitochondria-depolarizing agent carbonylcyanide *m*-chlorophenylhydrazone (CCCP) were used as positive control.

that the treatment per se did not render the PLTs prone to phagocytosis, as pHrodo fluorescence on monocytes incubated with acid-treated PLTs in the absence of antibodies was not increased (Fig. 6C).

Acid treatment protects PLTs from HLA antibody–mediated complement activation

The classical pathway of complement activation is another way by which anti-HLA can cause PLT destruction.[10] To evaluate whether acid-treated PLTs would be protected from killing via this pathway, an assay was established to measure, simultaneously, attachment of complement components C1q and C3c at the PLT surface and lysis of the PLTs. To detect lysis, PLTs were labeled with the cytosolic dye calcein red-orange that leaks out when the plasma membrane is damaged by the membrane attack complex.[27] Activation of the classical complement pathway was observed in samples incubated with plasma as a source of complement and HLA Class I

antibody together, as signified by deposition of both C1q and C3 on the PLT surface and leakage of calcein (Fig. 7A). The presence of complement alone led to binding of C1q to some of the PLTs, but the complement cascade was not activated on these PLTs, as neither the C3 component could be detected on their surface, nor did they lose the calcein fluorescence (Fig. 7A). Control-treated PLTs were lysed to the same extent as untreated PLTs, while acid-treated PLTs were completely protected (Figs. 7B and 7C). Of five tested plasma samples from patients with anti-HLA antibodies, only one activated the complement cascade. The reasons for the lack of complement activation in most cases are unknown. It was most likely caused by lack of appropriate HLA specificities in the tested sera, but difference in antibody isotypes could also play a role. When the complement cascade was activated, acid treatment reduced lysis, albeit not with the same completeness as seen for the MoAb (Fig. 7D).



PLT function remains intact several hours after the treatment

We next tested how acid treatment affected PLT function after 4 hours, representing a time period that most likely would pass before acid-treated PLTs could reach the patient. Four hours after treatment, there was still no difference in activation markers between acid-treated, control-treated, and untreated PLTs in response to TRAP-6 stimulation (Supplementary Fig. 3A, available as supporting information in the online version of this paper). Interestingly, the slight basal increase of activation markers on acid-treated PLTs persisted even after the longer resting time. The acid-treated PLTs' ability to aggregate was still intact 4 hours after treatment, also at this time point showing a stronger response to collagen compared to control-treated PLTs (Supplementary Fig. 3B).

DISCUSSION

Leukoreduction of blood products has reduced anti-HLA immunization and PLT refractoriness[7] and many blood centers have a pool of typed donors. Nevertheless, HLA antibody–mediated PLT refractoriness remains a clinical problem.[2] In a recent survey among 45 members of America's Blood Centers, lack of a pool of typed donors and the

Fig. 3. Acid treatment does not impair PLT activation. (A) Expression levels of PLT surface molecules on untreated, control-treated, and acid-treated PLTs were analyzed by flow cytometry. Relative expression levels to untreated PLTs from eight independent experiments and means are plotted. Differences between relative expression levels were not significant using the Holm-Sidak method with $\alpha = 5\%$. (▒) Control treated; (□) acid treated. (B) Expression of activation markers was analyzed on untreated, control-treated, and acid-treated PLTs in the resting state (gray histograms) or when stimulated with 20 μmol/L TRAP-6 for 20 minutes (black lines). (C) Mean values ± SD for activation marker expression from eight experiments performed within 1 hour after the treatment. (■) Untreated; (▲) control-treated; (△) acid-treated. Significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$. The indicated p values were obtained for the comparison of acid treated with untreated and acid-treated with control-treated.

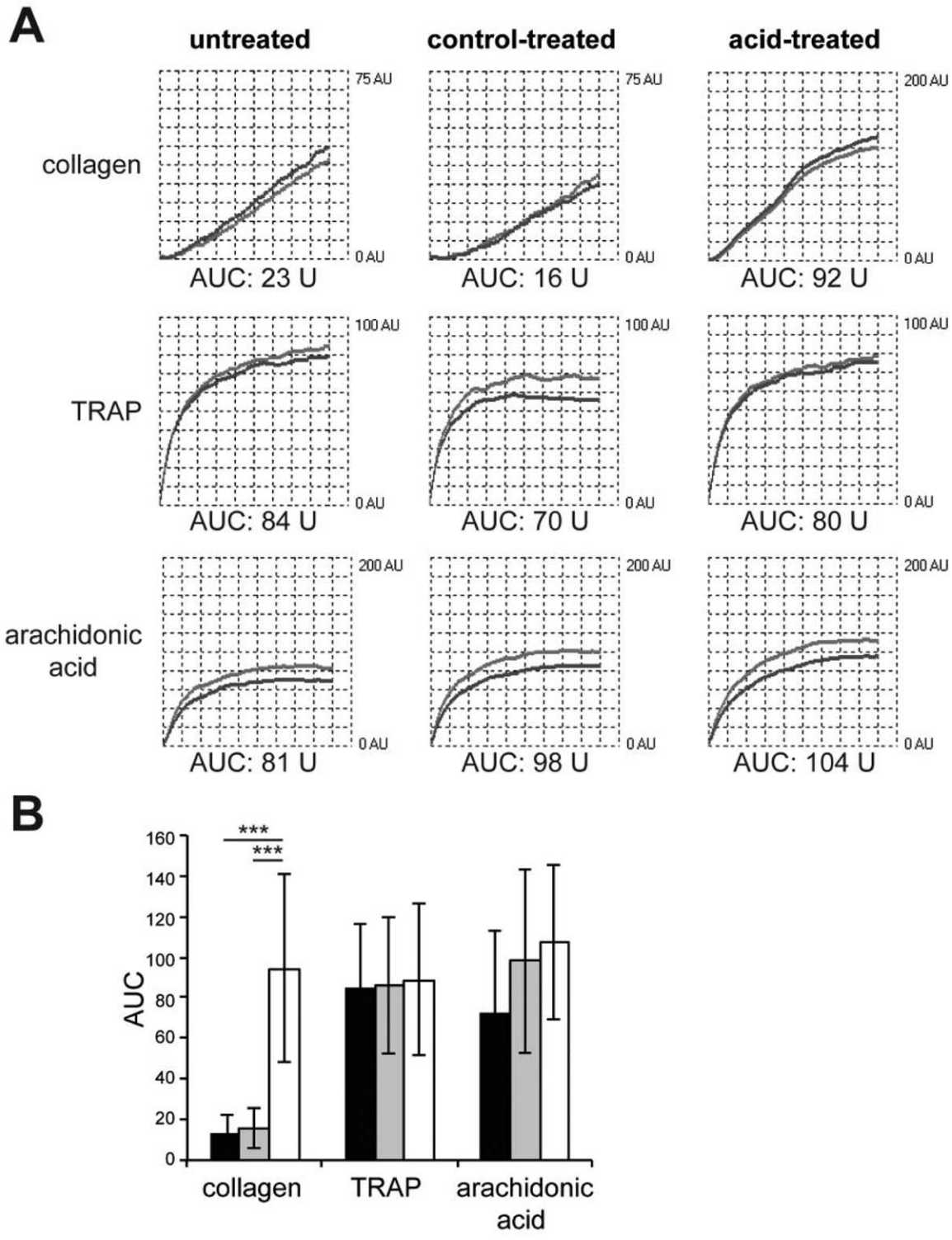


Fig. 4. Acid treatment does not impair PLT aggregation. (A) Representative aggregation curves of untreated, control-treated, and acid-treated PLTs stimulated with collagen, TRAP-6, or arachidonic acid measured with a multiplate analyzer. The two curves represent parallel measurements in the same sample; the area under curve (AUC) value is calculated from the mean of the two curves and given in arbitrary units. (B) Mean AUC values ± SD of 10 experiments. (■) Untreated; (▒) control-treated; (□) acid-treated. Significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test, ***$p \leq 0.001$.

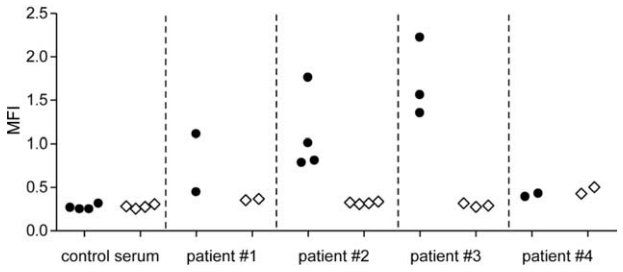


Fig. 5. Acid treatment prevents binding of anti-HLA. Untreated and acid-treated PLTs were incubated with EDTA plasma samples from HLA-immunized patients and IgG bound to the PLTs was detected by flow cytometry. Each dot represents PLTs from a single donor. The same donor was always tested against both untreated (●) and acid-treated (◇) PLTs.

time, cost, and logistics of obtaining appropriate units were named as the most common obstacles to providing appropriate PLTs to refractory patients.[28] Therefore, we believe that it is worthwhile to revive the idea of using acid-treated PLTs to improve the treatment of refractory patients.

Previous studies showed conflicting results regarding the efficiency of HLA removal and PLT viability and function after acid treatment. Resolving these issues and providing a comprehensive picture of acid treatment has been one key objective in our study. Three groups found that acid treatment of PLTs strongly reduced the amount of native HLA Class I on the surface and prevented binding of antibodies from the sera of immunized patients.[13,16,19] However, there was one study that found a high variability in the efficiency of the removal[20] and Murphy and coworkers[18] reported that their treatment protocol had only minor effects on binding of anti-HLA antibodies in a MoAb-specific immobilization of PLT antigens assay. The protocol applied in our study reliably removed 75 to 90% of native HLA Class I from the PLTs.

A controversial issue has been the viability and functionality of acid treated PLTs. Previous studies analyzing PLT morphology,[20,29] ATP content, and osmotic reversal[20] or the survival of indium-111–labeled acid-treated PLTs in healthy volunteers[14] have suggested that PLT viability is only moderately affected by acid treatment. We substantiated this conclusion using the MMP as a sensitive readout for maintenance of oxidative phosphorylation capacity, loss of which is an early marker for early apoptotic processes. In terms of functional consequences, results have been conflicting. Kurata and colleagues[13] found only slightly reduced aggregation responses to different stimuli after exposure to low pH. In contrast, Murphy and colleagues observed an almost complete loss of function.[18] In our study, acid-treated PLTs responded well in multiplate analysis to all tested stimuli, with no evidence of reduction in aggregation. Interestingly, we observed an increased aggregation response of acid-treated PLTs to collagen stimulation. This was not due to enhanced cell surface expression of the $\alpha_2\beta_1$ integrin, a major collagen receptor on PLTs. It is possible, however, that acid treatment triggers a conformational change of the $\alpha_2\beta_1$ integrin to the high-affinity form, similar to what happens to the fibrinogen receptor after PLT activation, which could lead to an increased response. Further studies are needed to investigate this possibility, also taking into account effects of acid treatment of GPVI, the other major collagen receptor on PLTs.

One point that our study has addressed for the first time is whether the acid treatment protects the PLTs from antibody-mediated destruction mechanisms. Binding of the MoAb W6/32 was not completely abrogated after the treatment, which could be explained by incomplete denaturation of HLA complexes or the low affinity of the antibody to free heavy-chain molecules.[30] Even though we did not find residual binding of antibodies from the patient samples tested (Fig. 5), it is possible that polyclonal antibodies of some patients could bind to acid-treated PLTs, albeit to a lower extent. The lower binding of W6/32 to acid-treated PLTs strongly reduced phagocytosis by monocytes in our in vitro assay. One has to take into account that a mouse MoAb might not have the full potential of a human antibody to elicit phagocytosis and that other phagocytes like neutrophils or splenic macrophages may have lower thresholds for phagocytosis of opsonized PLTs than peripheral blood monocytes.

Complement activation can enhance phagocytosis of antibody-coated PLTs by stimulation of complement receptors on phagocytes, but can also lead to complement-mediated lysis of opsonized cells. We found that acid-treated PLTs were protected from both deposition of activated complement components on the surface and complement-mediated lysis in the presence of HLA antibodies. When we used the W6/32 antibody, the protection was complete despite the residual binding we observed. We assume that the density of bound antibodies on the surface of acid-treated PLTs is below the threshold needed for complement activation. When we used the serum of a refractory patient, lysis was not prevented completely. Even though the protection was not complete, the results of both assays suggest that acid treatment has a good chance to prolong the survival of transfused PLTs in refractory patients.

While efficiently stripping off classical HLA Class I antigens, it cannot be excluded that the denaturing effect of acid treatment could expose neoantigens on the PLTs that might induce antibodies specific for acid-treated PLTs. This question can only be addressed in humans when a sufficiently large number of patients have been treated. Meanwhile, the immunogenicity of acid-treated PLTs could be studied in mouse models by monitoring

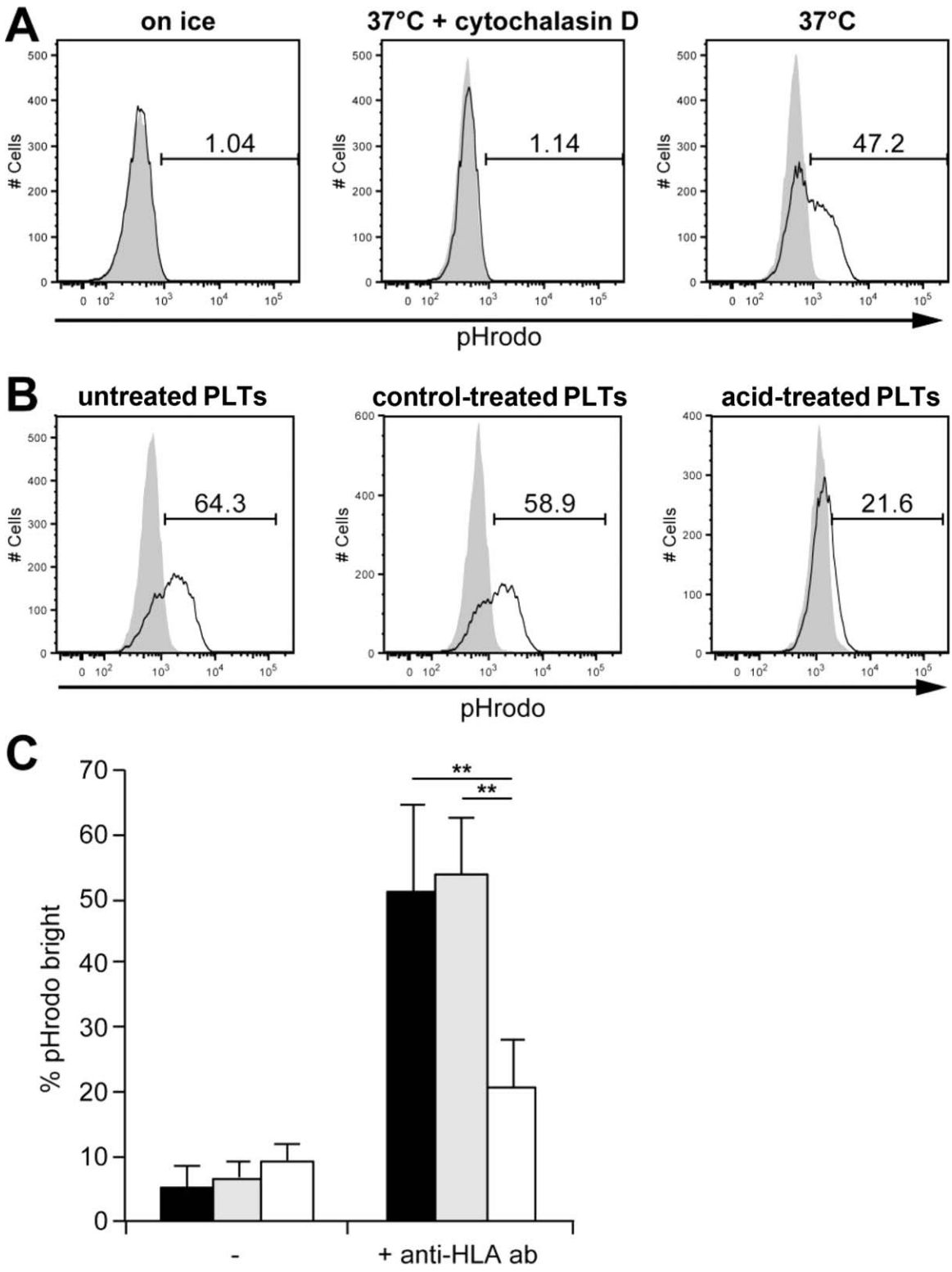


Fig. 6. Acid treatment protects PLTs from HLA antibody–mediated phagocytosis. (A) PBMNCs were incubated with PLTs labeled with pHrodo alone (gray histograms) or coated with anti-HLA MoAb (black line) for 2 hours under the indicated conditions. Events are gated on monocytes and the numbers represent the percentage of monocytes with high pHrodo fluorescence intensity in the samples with the antibody. (B) PBMNCs were incubated with pHrodo-labeled PLTs that had been treated as indicated and phagocytosis was analyzed as before. The gate for the pHrodo-bright population was set according to the cytochalasin D–treated samples. (C) Mean values ± SD of four experiments including six combinations of two donors and four PLT concentrates. (■) Untreated PLTs; (▒) control-treated PLTs; (□) acid-treated PLTs. Significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test, $^{**}p \leq 0.01$.

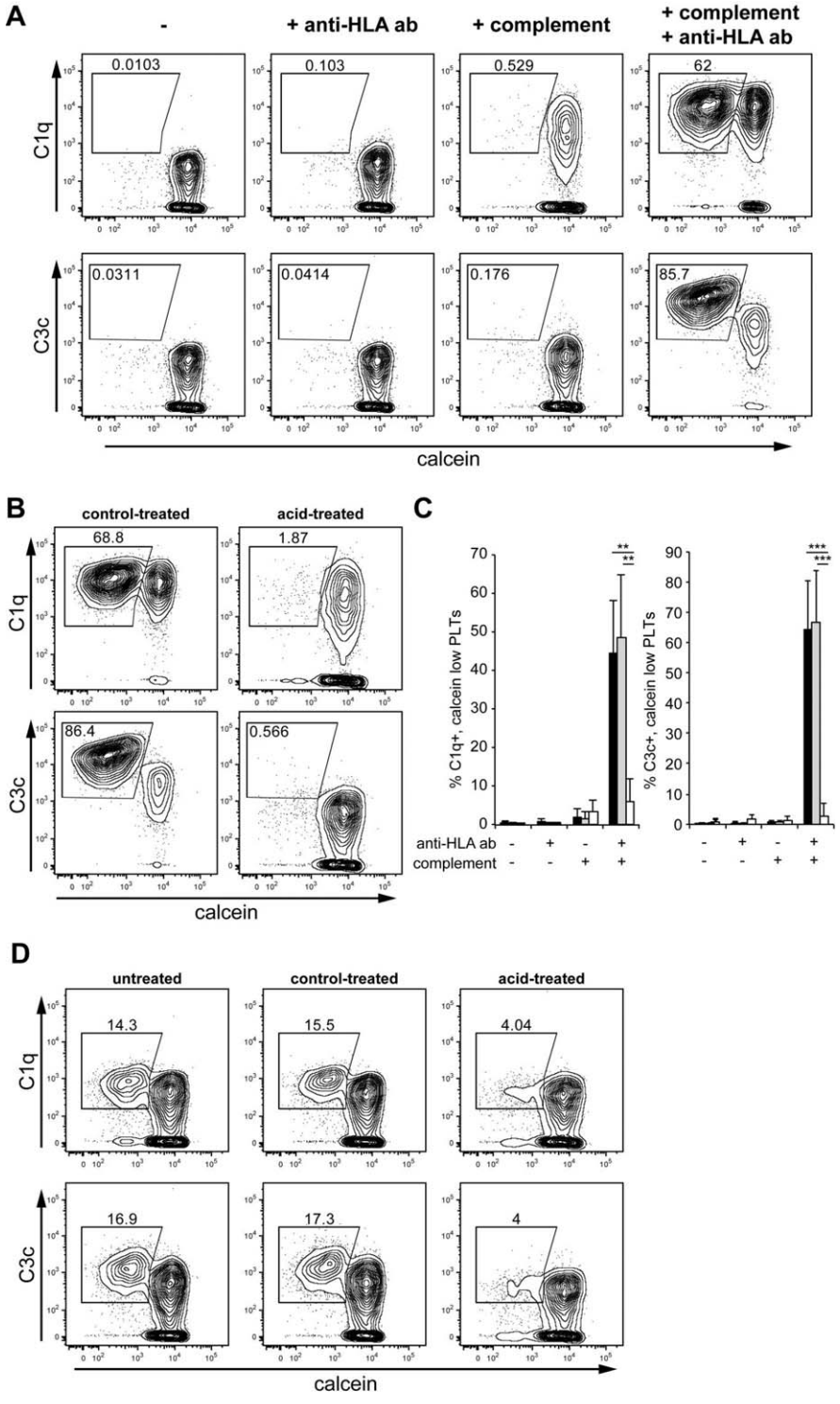


Fig. 7. Acid treatment prevents HLA antibody–mediated lysis by complement. (A) Calcein-labeled PLTs were incubated for 10 minutes at 37°C in the absence or presence of a monoclonal anti-HLA-A,B,C antibody and plasma containing active complement. Complement-mediated PLT lysis was assessed by staining for complement components C1q or C3c on the PLT surface and calcein leakage. The numbers indicate percentage of events in the gate. (B) Representative plot of control- or acid-treated PLTs incubated with HLA antibody and plasma for 10 minutes and stained for C1q or C3c. (C) Mean percentages of calcein low PLTs positive for C1q or C3c ± SD for five to six experiments. (■) Untreated; (▒) control treated; (□) acid treated. Significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. (D) Untreated, control-treated, and acid-treated PLTs were incubated for 10 minutes with serum of an HLA-immunized patient in the presence of active complement and complement-mediated PLT lysis was assessed as previously.

survival and function of acid-treated PLTs after repeated transfusions. Animal models could also be used to study in more detail how acid-treated PLTs escape from phagocytosis and complement-mediated destruction in vivo, as well as for preclinical studies of PLT function after acid treatment, allowing kinetic estimations of PLT survival in comparison with allogeneic normal PLTs.

Our in vitro findings suggest that acid-treated PLTs could be used as an alternative to HLA-matched PLT transfusions. So far, we have verified normal function up to 4 hours after acid treatment, a minimum time expected to pass before treated PLTs would reach the patient. A longer posttreatment storage study would be useful to indicate flexibility in terms of when treatment can be done in relation to the planned transfusion time. Our results open up for a clinical trial, in which the potential of acid-treated PLTs in refractory patients are studied. The relatively simple method of acid stripping has the potential to strongly improve the treatment of refractory patients, especially in acute situations when no matched PLTs are available, but also saving time and costs in prophylactic care compared to HLA-matched donor PLTs.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Supplementary Fig. 1. Representative histograms of platelet diameter distribution after 5 min treatment. Platelet size distribution was measured using a CASY cell counter. Representative histograms of platelet diameter distribution after 5 minutes treatment are shown. The inset mean values of platelet diameter ± SD are from the indicated numbers independent experiments.

Supplementary Fig. 2. The principle of the pHrodo-based phagocytosis assay.

Supplementary Fig. 3. Platelet function is intact four hours after the acid treatment. (A) Expression of activation markers was analyzed on resting and TRAP-6-stimulated platelets 4 hours after treatment. Mean values ± SD from six experiments. (B) Platelet aggregation in response to the indicated stimuli was measured 4 hours after treatment using a multiplate aggregometer. Mean AUC values ± SD of eight experiments. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.