



HLA class I depletion by citric acid, and irradiation of apheresis platelets for transfusion of refractory patients

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Abstract

Background: Patients can form antibodies to foreign human leukocyte antigen (HLA) Class I antigens after exposure to allogeneic cells. These anti-HLA class I antibodies can bind transfused platelets (PLTs) and mediate their destruction, thus leading to PLT refractoriness. Patients with PLT refractoriness need HLA-matched PLTs, which require expensive HLA typing of donors, antibody analyses of patient sera and/or crossmatching. An alternative approach is to reduce PLT HLA Class I expression using a brief incubation in citric acid on ice at low pH.

Methods and Materials: Apheresis PLT concentrates were depleted of HLA Class I complexes by 5 minutes incubation in ice-cold citric acid, at pH 3.0. Surface expression of HLA Class I complexes, CD62P, CD63, phosphatidylserine, and complement factor C3c was analyzed by flow cytometry. PLT functionality was tested by thromboelastography (TEG).

Results: Acid treatment reduced the expression of HLA Class I complexes by 71% and potential for C3c binding by 11.5-fold compared to untreated PLTs. Acid-treated PLTs were significantly more activated than untreated PLTs, but irrespective of this increase in steady-state activation, CD62P and CD63 were strongly upregulated on both acid-treated and untreated PLTs after stimulation with thrombin receptor agonist peptide. Acid treatment did not induce

Abbreviations: FITC, fluorescein isothiocyanate; HLA, human leukocyte antigen; MA, max amplitude; MFI, median fluorescence intensity; MPV, mean platelet volume; PE, phycoerythrin; PFA, paraformaldehyde; PLTs, platelets; TEG, thromboelastography; TRAP6, thrombin receptor activator peptide 6

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apoptosis over time. X-ray irradiation did not significantly influence the expression of HLA Class I complexes, CD62P, CD63, and TEG variables on acid treated PLTs.

Conclusion: The relatively simple acid stripping method can be used with irradiated apheresis PLTs and may prevent transfusion-associated HLA sensitization and overcome PLT refractoriness.

KEYWORDS

hematology – platelets, immune thrombocytopenia, platelet transfusion

1 | INTRODUCTION

Platelet (PLT) refractoriness represents a serious problem for patients who are PLT transfusion dependent. It is associated with adverse clinical outcomes, for example, increased bleeding risk, decreased survival, and increased health care costs due to increased length of hospital stay.¹ Refractoriness can be caused by antibodies against non-self human leukocyte antigen (HLA) Class I molecules.^{2,3} Previous transplantations, pregnancies, and blood transfusions are risk factors for HLA immunization.^{4,5} A majority of PLT-refractory patients are women with a hematologic diagnosis.⁶ Routine use of leukoreduced PLT concentrates reduces the incidence of HLA alloimmunization by more than 50%.⁷ However, from 2%-3% to 20%-30% of patients, depending on diagnosis and earlier pregnancies, who receive multiple PLT transfusions still develop HLA alloantibodies.⁷⁻⁹

The current standard approach to overcome PLT refractoriness caused by anti-HLAs is transfusion with HLA-matched PLTs,¹⁰ which is complicated and logistically demanding,¹⁰ especially in cases of emergency. An alternative approach to handle PLT refractoriness is to deplete or denature HLA Class I molecules on PLTs before transfusion.¹¹⁻¹³ Treatment with citric acid (pH 2.9-3.0) leads to denaturation of the trimolecular HLA Class I complexes without significant damage to the PLTs.¹³ Recently, Meinke et al¹³ reported that acid-treated PLTs derived from buffy coat have the potential to survive with intact functional capacity if transfused to PLT-refractory patients with anti-HLA Class I.

Irradiation of PLTs is commonly used with either gamma or X-rays before transfusion to prevent transfusion-associated graft-vs-host disease. This is applied to HLA-matched PLTs as well, if other measures such as pathogen inactivation¹⁴ are not undertaken. Transfusion-associated graft-vs-host disease is almost always fatal, with a less than 10% survival rate.¹⁵ Several publications have shown that X-ray irradiation is equivalent to gamma irradiation for inactivation of white blood cells.¹⁶⁻¹⁸

Thromboelastography (TEG) has become an ideal monitoring tool for blood transfusion management in severe surgery, trauma, leukemia, and hemophilia¹⁹ and has also been extensively used to characterize the properties of PLT concentrates in vitro.^{20,21} We used TEG analysis as a functional test after both the acid treatment and irradiation of the PLTs.

Thus, we have investigated the effect of acid treatment on apheresis PLTs and the effect of X-ray irradiation on acid-treated apheresis PLTs. Our data indicate that acid treatment can effectively reduce HLA Class I and β_2 -microglobulin expression and prevent classical complement activation on apheresis PLTs. X-ray irradiation seems to have no negative effect on PLT pH, mean PLT volume (MPV), activation markers (CD62P, CD63), and hemostatic function, as evaluated by TEG. The effect of acid treatment on anti-HLA binding capacity was evaluated by flow cytometry. Patient sera containing HLA Class I antibodies were tested against untreated and acid-treated PLTs.

2 | MATERIALS AND METHODS

2.1 | PLT collection by apheresis

Leukoreduced PLT apheresis concentrates were obtained from qualifying, healthy volunteer donors, using an automated blood cell separator, either a Trima (Gambro BCT, Lakewood, CO) or AMICUS (Baxter Fenwal Division, Deerfield, IL) apheresis machine (n = 5). Acid-citrate-dextrose formula A was used as anticoagulant during procedures according to the manufacturer's recommendations.

2.2 | Antibodies to cell surface molecules

For flow cytometry analysis these monoclonal antibodies from BioLegend (San Diego, California) were used: Fluorescein isothiocyanate (FITC)-conjugated mouse anti-

human HLA-A, -B, and -C, clone W6/32 (311404); purified anti-human HLA-A, -B, and -C, clone W6/32 (311402); and phycoerythrin (PE)-conjugated mouse anti-human β 2-microglobulin, clone 2M2 (316306). Adequate isotype controls were FITC-conjugated mouse anti-mouse NK1.1, clone PK136 (553164), and PE-conjugated mouse IgG1, κ (555750), respectively. For activation studies we used PE-Cy5-conjugated mouse anti-human CD62P clone AK-4 (556020), isotype control PE-Cy5-conjugated mouse IgG1 κ , and PE-conjugated mouse anti-human CD63 clone H5C6 (551142) with isotype control PE-conjugated mouse IgG1, κ as (555750) (BD PharMingen, San Diego, California). Expression of cell surface molecules was analyzed on a flow cytometer (Gallios, Beckman Coulter, Inc., Fullerton, California). The monoclonal anti-CD42a, eFluor 450-conjugated (48-0428-42) was from eBiosciences (San Diego, California). The polyclonal rabbit anti-human C3c complement/FITC (F0201) was from Agilent (Dako) (Santa Clara, California) and rabbit IgG isotype control, FITC (11-4614-80) was from eBiosciences (San Diego, California).

2.3 | Reagents

Thrombin receptor activator peptide 6 (TRAP6, T1573) and tetrapeptide Arg-Gly-Asp-Ser (RGDS, A9041) were from Sigma-Aldrich (St. Louis, Missouri). Phosphate-buffered saline (PBS; Gibco Life Technologies, 10 010 056; Rockville, Maryland), paraformaldehyde (PFA, 8.18175), citric acid monohydrate (1002440500) and disodium hydrogen phosphate dihydrate (106580) were from Merck (Darmstadt, Germany). Apotracker Green kit (427401) was from BioLegend (San Diego, California).

2.4 | Acid treatment of apheresis platelets

Protocol for the acid treatment was modified from that of Meinke et al.¹³ PLTs from apheresis concentrates (420-500 mL and $1-1.5 \times 10^9$ PLTs/mL) were split in two and transferred to two transfer bags (600 mL, Compoflex R6R2021; Terumo BCT, Lakewood, Colorado) 24 hours after processing and centrifuged ($1506 \times g$, 10 minutes, accel 8, decel 2, temperature 22°C). One half of the initial product was acid treated, the other served as control. The PLT poor plasma was transferred to new bags by an automatic press (Optipress, Fenwal). To avoid transfer of untreated PLTs to the final product, the satellite bags with PLT-poor plasma were recentrifuged ($2524g$, 14 minutes, accel 9, decel 5, at 22°C) to obtain PLT-free plasma, for later resuspension of the acid-treated PLTs and the untreated PLTs, respectively. The PLT pellets (about

10 mL) were gently blended with the rest of the plasma to dissolve PLT aggregates. The pellets were precooled on ice for 2 minutes. To one pellet kept on ice, 30 mL of ice-cold citric acid buffer (equal volumes of 263 mmol/L citric acid and 123 mmol/L Na_2HPO_4 , resulting in pH 2.9-3.0) was added by syringe in a sterile safety cabinet (model NU-201-430e, NuAire Lab Equipment, Plymouth, Minnesota), and content mixed gently thrice. After 5 minutes of incubation on ice, the acid was neutralized in excess volume (20-fold) of PLT Additive Solution (T-PAS+, 18359006; Terumo BCT). PLTs were centrifuged, supernatant removed, pellets, dissolved by gently mixing, and PLTs were resuspended in their original, now PLT-free plasma. Control PLTs were treated by the exact same procedure, except for the acid treatment. Control and acid-treated PLT concentrates were kept unagitated for 30 minutes followed by 30 minutes on a horizontal flatbed PLT agitator (Helmer Laboratories, Inc., Noblesville, Indiana).

2.5 | Analysis of pH, MPV, and PLT count measurement

PLT suspension (1.5 mL) was collected into a blood gas syringe (safePICO; Radiometer, Copenhagen, Denmark) and pH measured using routine blood gas equipment (Cobas b221; Radiometer, Copenhagen, Denmark). pH of all samples was measured at 22°C.

PLT count and MPV measurements were performed on a standard hematology analyzer (ABX Pentra XL80, HORIBA Diagnostics, Kyoto, Japan). To estimate loss of PLTs during treatment, the percentage of PLT recovery was calculated as PLT count in acid-treated PLTs or control PLTs divided with PLT count before acid treatment $\times 100$. MPV was measured on Days 1 and 2.

2.6 | X-ray irradiation

One part of the acid-treated or untreated product was subjected to X-ray irradiation for 4.2 minutes with 25 Gy (Raycell Mk2 X-ray Blood Irradiator, Best Theratronics', Ottawa, Canada).

2.7 | Cell surface staining of HLA Class I molecules and β 2-microglobulin

PLTs were diluted 1:50 with PBS and 5 μL of FITC-conjugated mouse anti-human HLA-A,-B, or -C or PE-conjugated mouse anti-human β 2-microglobulin clone 2M2 were added to 100- μL cell suspension. Isotype controls were run in parallel. Samples were incubated for 30 minutes at room temperature in the dark, resuspended

in 400 μL of PBS, and analyzed by flow cytometry within 1 hour. For the calculation of percent reduction of HLA Class I molecules and $\beta 2$ -microglobulin expression levels, the median fluorescence intensity (MFI) of samples incubated with the isotype control was subtracted from the MFI of PLTs stained with anti-human HLA-A, -B, or -C and anti-human $\beta 2$ -microglobulin and the corrected MFIs of treated sample were divided by the MFI of the corresponding untreated sample $\times 100$.

2.8 | Complement activation assay

One hundred microliters of untreated, acid-treated, and acid-treated and irradiated PLTs ($0.8\text{--}1.0 \times 10^6/\mu\text{L}$) were added to 500 μL of PBS mixed 1:1 with normal or heat-inactivated (56°C , 30 minutes) human plasma, with or without 5 $\mu\text{g}/\text{mL}$ mouse anti-human HLA-A, -B, or -C antibody and incubated for 10 minutes at 37°C . Fifty microliters of each sample were thereafter stained for CD42a and C3c or isotype control and analyzed by flow cytometry, to measure HLA antibody-antigen-mediated classical complement activation, compared to negative controls without HLA antibody.

2.9 | Cell surface staining of activation markers

We examined the surface expression of CD62P and CD63 on resting PLTs to evaluate spontaneous PLT activation and stimulated PLTs as a measure of the residual PLT activation potential.²² Tetrapeptide RGDS (4.6 mmol/L final concentration) was added to the PLTs and incubated for 10 minutes at 20°C , to prevent aggregation before stimulation with 0.4 mmol/L (final concentration) of TRAP6 for 20 minutes at 20°C . Stimulated and resting PLTs were fixed with 0.5% (final concentration) PFA in PBS, placed on ice for 20 minutes, and diluted 1:10 with PBS with 5.6 mmol/L glucose and 3.5 g/L bovine serum albumin. Suspensions of resting and stimulated PFA-fixed PLTs (25 μL) were then incubated 20 minutes in the dark at 20°C with either 10 μL of PE-Cy5 mouse anti-human CD62P or PE-Cy5 mouse IgG1 κ isotype control, PE mouse anti-human CD63, or PE mouse IgG1, κ isotype control. After incubation, 500 μL of ice-cold 1% PFA were added for fixation, and flow cytometric analysis was performed within 30 minutes.

2.10 | Flow cytometric analysis of phosphatidylserine exposure on PLTs

To assess phosphatidylserine (PS) on the surface of PLTs, 5 μL of Apotracker Green were added to suspensions of 1×10^6 untreated, acid-treated, and acid-treated and

irradiated apheresis PLTs, and incubated for 25 minutes at 20°C . The analysis was performed using flow cytometry.

2.11 | TEG test

A hemostasis system (TEG Hemostasis System, model 5000; Haemonetics Corp., Braintree, Massachusetts) and auxiliary reagents were used to evaluate coagulation function according to the manufacturer's instruction. Apheresis PLTs were diluted $1:10^{21}$ in AB Octaplas. Briefly, 1 mL of the diluted apheresis PLTs was added to a prewarmed vial with kaolin (TEG Hemostasis System, Kaolin Reagent 6300), and mixed by inversion. Immediately, 340 μL of this suspension were pipetted into a 37°C prewarmed analyzer cup containing 20 μL 0.2 M CaCl_2 and the analysis was initialized. The test measures thrombin-activated clotting and was run until the following parameters were defined: Reaction time (R-time), the period from the sample was placed in the instrument until the first fibrin is formed, reflects the level of coagulation factors present. Clot kinetics (K time) reflects the time until the clot reaches a fixed strength. α angle is how steep the curve is rising and reflects the speed of fibrin accumulation and the level of fibrinogen. Max amplitude (MA) is the maximum distance between the curve lines and reflects number and function of the platelets, level of fibrinogen, and strength of the formed clot.

2.12 | HLA antibody binding tested by flow cytometry

Twelve anonymized samples from PLT-refractory patients in the hematology department, all known to contain multiple anti-HLA Class I antibodies of various specificities, were tested against different PLT concentrates, with known HLA Class I molecules, all expected to be incompatible with the patient sera.

The patient sera were incubated with suspensions of PLTs from acid-treated and untreated PLTs from the same donor at 37°C for 40 minutes and washed twice. A secondary antibody, goat anti-human IgG/FITC (109-096-088, Jackson Immuno Research, Ely, UK) was added and incubated for 40 minutes at 20°C , before performing flow cytometric analysis.

The known specificities tested, included antibodies against the HLA-A1, -A2, -A3, -A24, -B7, -B8, -B44, -B51, -B57, and -B62.

2.13 | Statistical analysis

Data are presented as mean \pm standard deviation (SD). Student's *t* test was used to evaluate the statistical

significance of the results. Differences with P values less than .05 were considered significant.

3 | RESULTS

3.1 | Acid treatment of apheresis PLTs reduces the expression of HLA Class I molecules and β_2 -microglobulin

Our results showed that the acid treatment reduced the expression of HLA Class I molecules by 70.6% (± 8.1) and

β_2 -microglobulin by 74.2% (± 9.4) as compared to untreated PLTs, respectively (Figure 1A, B). For PLTs that were irradiated after acid treatment, HLA Class I protein expression was reduced by 72.8% (± 8.4) and β_2 -microglobulin by 75.6% (± 7.8). Thus, irradiation did not significantly affect the reduction.

When comparing complement activation ability after the addition of anti-HLA Class I, C3c expression on the surface of acid-treated and acid-treated and irradiated PLTs decreased 11.5- to 12.0-fold compared with that of the untreated PLTs. This must be due to removal and denaturation of the HLA antigens on the PLT surface (Figure 2A and B). Also, as

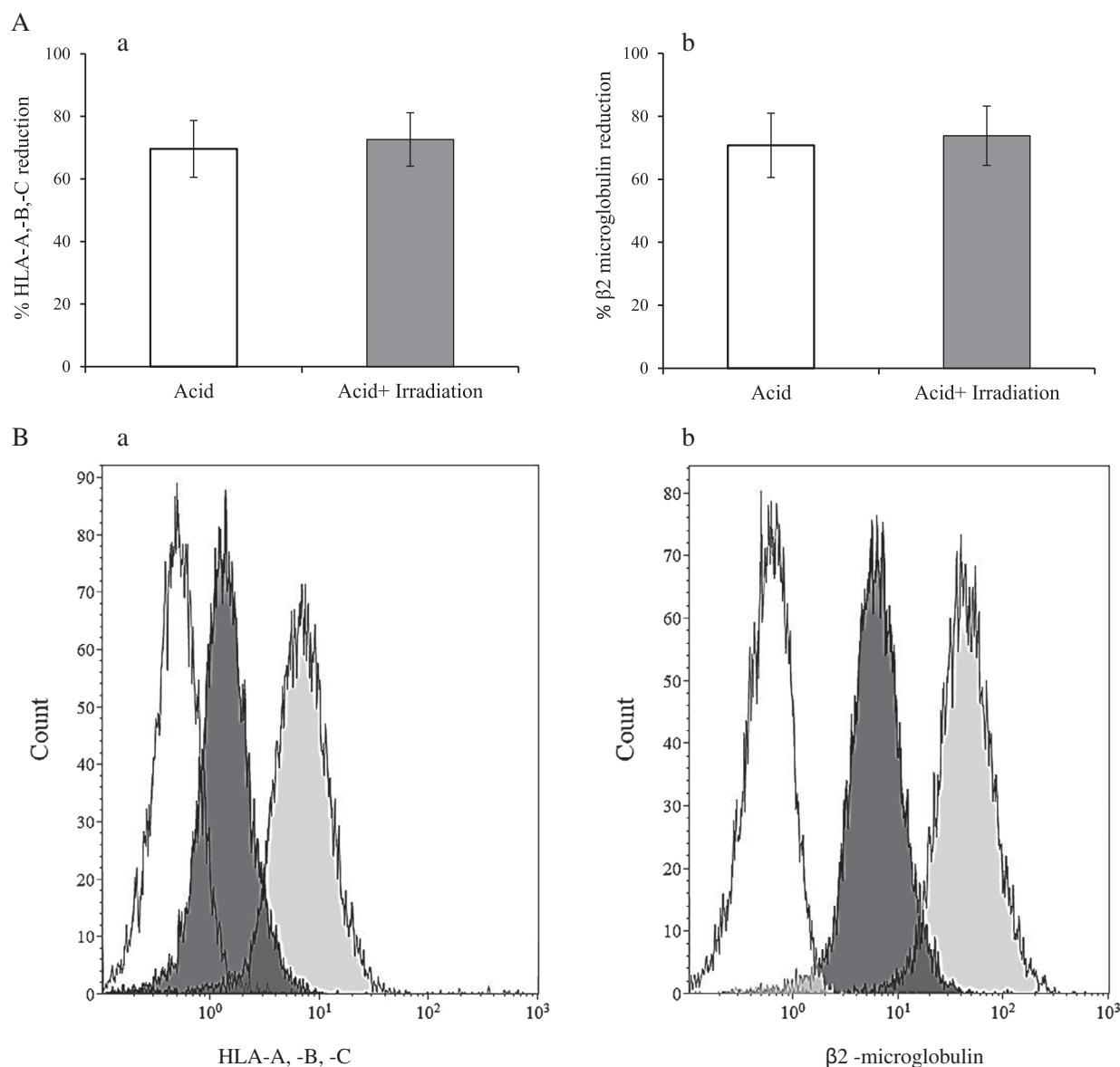


FIGURE 1 (A) Effect of acid treatment and irradiation on (a) HLA-A, -B, and -C, and (b) β_2 microglobulin expression on acid treated apheresis PLTs. The reduction of HLA-A, -B, and -C and β_2 -microglobulin is measured as percentage of reduction of acid-treated comparing to untreated control. Results are given as mean \pm SD ($n = 5$). (B) Representative histograms for surface staining of HLA-A, -B, and -C (a) and β_2 -microglobulin (b). White peaks represent isotype controls, black peaks represent results after 5 minutes of acid treatment on ice, and gray peaks represent results after 5 minutes on ice, of untreated PLTs. Student's t test was used to evaluate the statistical significance of the results with P values $< .05$

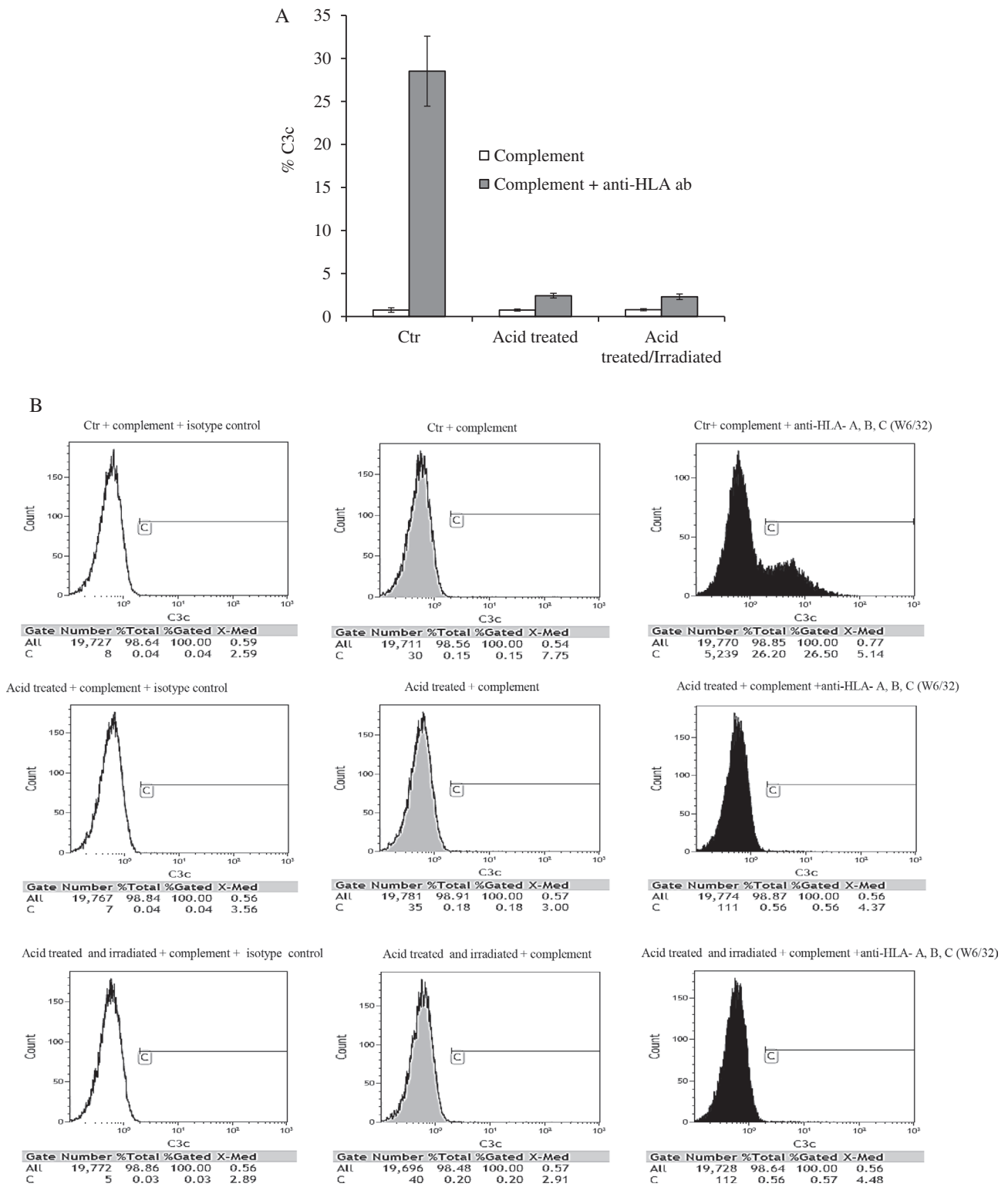


FIGURE 2 (A) Effect of acid treatment on antibody-mediated complement activation. Surface expression of complement component C3c was measured on untreated, acid treated and acid treated/irradiated PLTs, after incubation with fresh plasma (complement source) and anti-HLA-A, -B, and -C antibody. Control with heat-inactivated plasma + anti-HLA, irrelevant isotype control, and control without HLA antibody were run in parallel. The reduction of C3c on the surface of the PLTs is an expression of missing antibody-binding due to the depletion and denaturation of HLA antigens. Results are given as mean \pm SD (n = 4). (B) Representative histograms for surface staining of C3c. Untreated/ctr, acid-treated and acid-treated and irradiated apheresis PLTs were incubated with plasma (complement source) or plasma and anti-HLA antibody for 10 minutes and stained for C3c. Adequate controls were included. Gate C represents percentage of C3c positive cells. White peaks represent isotype controls. Gray peaks represent complement and black peaks represent complement + anti-HLA

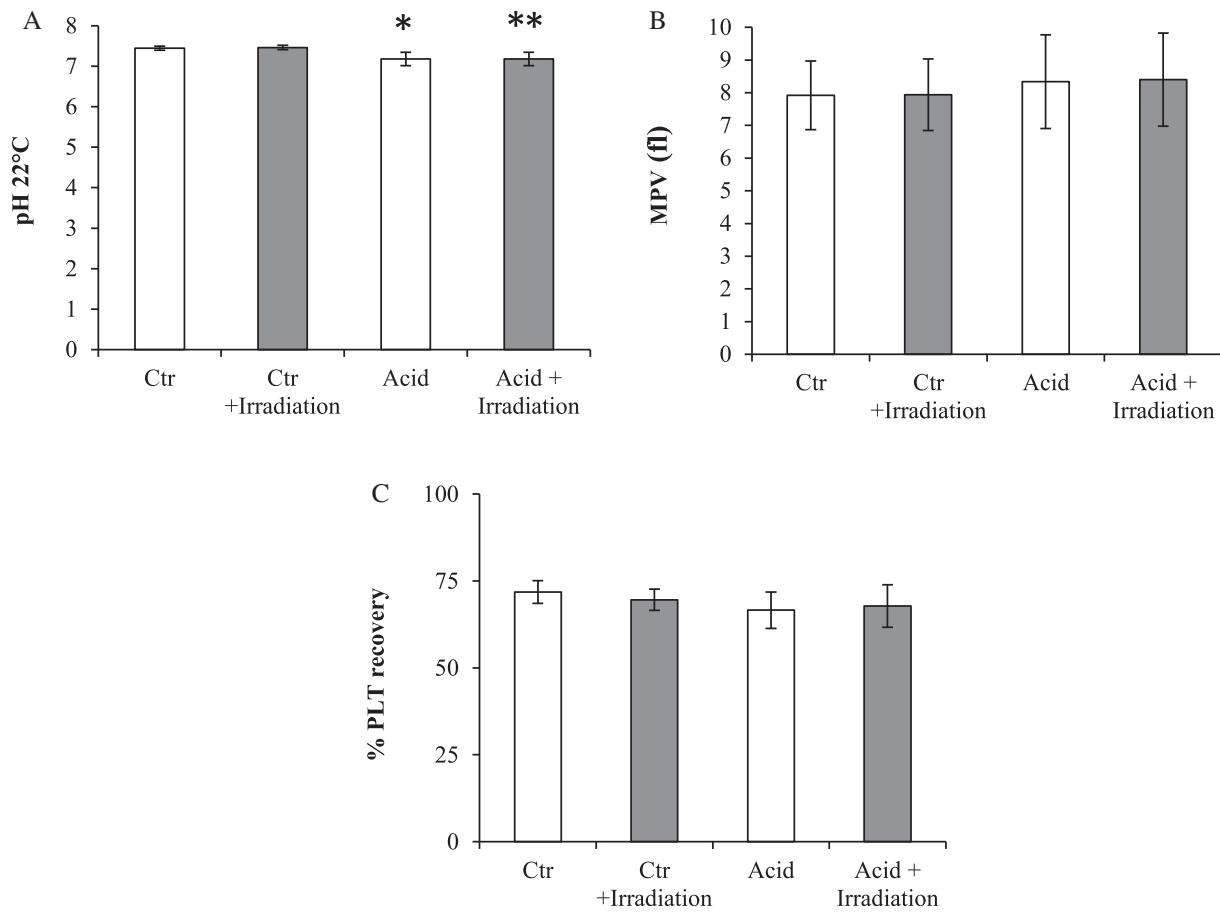


FIGURE 3 Effect of acid treatment and irradiation on pH (A), MPV (B) and % PLT recovery (C) of apheresis PLTs. The untreated PLTs or acid treated PLTs were subjected to X-ray irradiation for 4.2 minutes with 25 Gy. Results are given as mean \pm SD ($n = 5$). Student's *t* test was used to evaluate the statistical significance of the results with *P* values $< .05$. **P* in comparison with acid-treated and **in comparison with acid-treated and irradiated PLTs

expected, complement expression was nonexistent, in the control, where heat-inactivated plasma was included.

3.2 | Acid treatment of apheresis PLTs reduces pH but has insignificant effect on MPV and recovery

The pH value is an important marker for in vitro quality of PLT concentrates.²³ Acid treatment of apheresis PLTs significantly decreased pH to 7.18 (± 0.16) as compared to untreated controls of 7.44 (± 0.05). PLT concentrates that were irradiated after acid treatment had a pH of 7.18 (± 0.16) vs untreated, irradiated ones of pH 7.46 (± 0.05). Hence, pH was unaffected by irradiation (Figure 3A).

Acid treatment did not significantly change MPV, as untreated PLTs had MPV of 7.9 (± 1.1) and acid-treated PLTs had MPV of 8.3 (± 1.4). Also, irradiation did not significantly affect MPV of acid-treated apheresis PLTs. The

results were 8.4 (± 1.4) for acid-treated and 7.9 (± 1.1) for untreated PLTs (Figure 3B). Furthermore, acid treatment did not significantly change MPV after 48 hours (Figure S1).

Acid treatment did not significantly change percentage of PLT recovery; 69.6% (± 5.2) as compared to untreated control of 71.8% (± 3.3) (Figure 3C). Irradiation did not significantly affect percentage of PLT recovery of acid-treated apheresis PLTs that was 67.8% (± 6.1) (Figure 3C).

3.3 | Acid treatment of apheresis PLTs does not change the residual activation potential

We examined the surface expression of CD62P and CD63 on acid-treated apheresis PLTs to evaluate spontaneous PLT activation. There was a small basic increase of CD62P expression on acid-treated PLTs (MFI = 227 \pm 52)

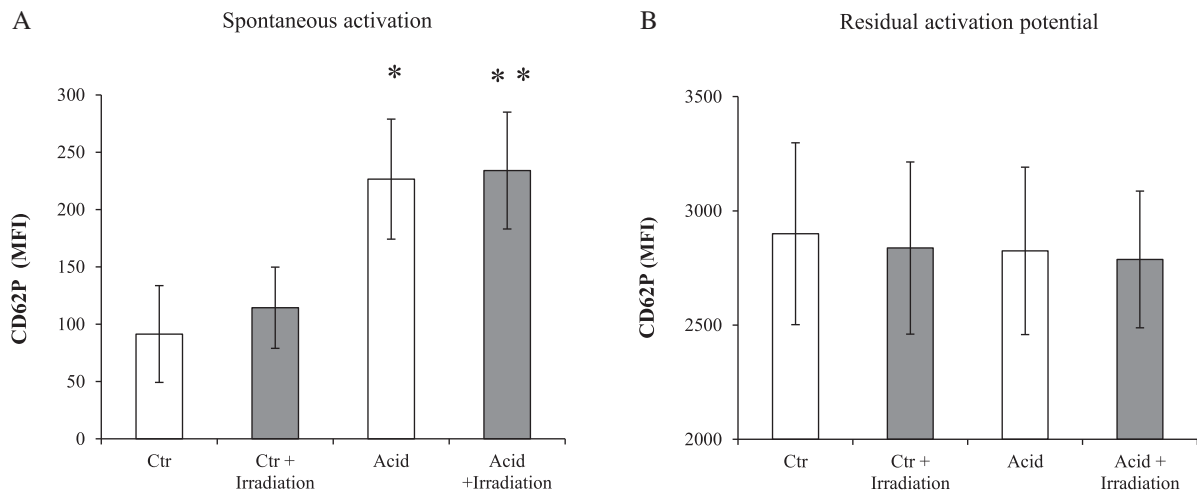


FIGURE 4 Effect of acid treatment and irradiation on spontaneous activation (A) and residual activation potential (B) as measured by CD62P expression on apheresis PLTs. Median fluorescence intensity (MFI) is measured by flow cytometry. Results are given as mean \pm SD ($n = 5$). Student's *t* test was used to evaluate the statistical significance of the results with *P* values $<.05$. **P* values $<.05$ in comparison with acid-treated and **in comparison with acid-treated and irradiated PLTs

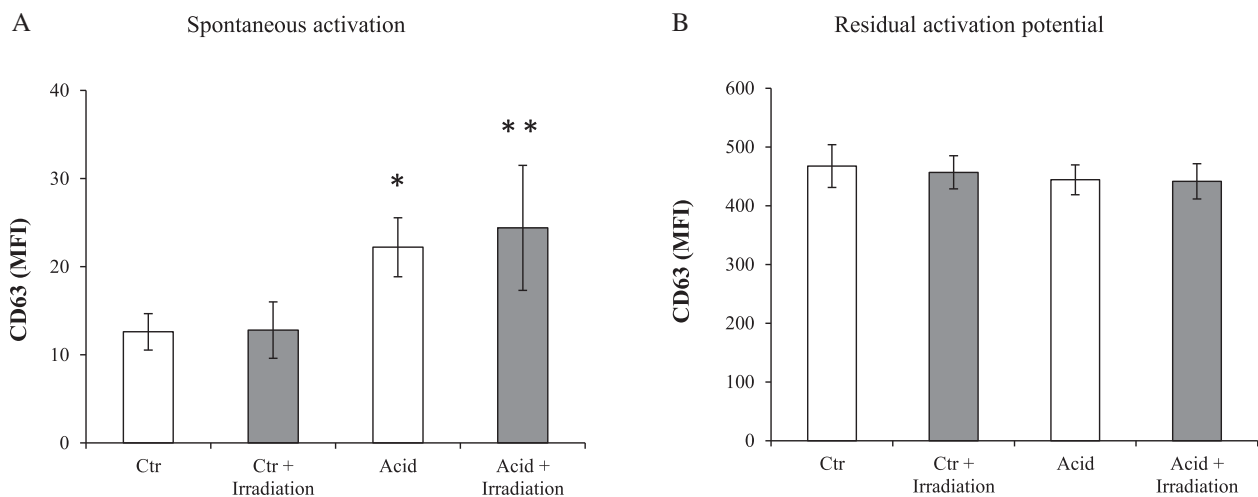


FIGURE 5 Effect of acid treatment and irradiation on spontaneous activation (A) and residual activation potential (B) as measured by CD63 expression on apheresis PLTs. Median fluorescence intensity is measured by flow cytometry. Results are given as mean \pm SD ($n = 5$). Student's *t* test was used to evaluate the statistical significance of the results with *P* values $<.05$. **P* values $<.05$ in comparison with acid-treated and ** in comparison with acid-treated and irradiated PLTs

as compared to untreated PLTs (MFI = 91 ± 42) (Figure 4A). CD63 expression increased from MFI = $13 (\pm 2)$ in untreated PLTs to MFI = $22 (\pm 3)$ on acid-treated PLTs (Figure 5A).

In contrast, CD62P and CD63 were strongly upregulated after stimulation with TRAP6 on both acid-treated and untreated PLTs (Figures 4B, 5B and 6). Thus, no significant difference was found in the residual activation potential. The X-ray irradiation had no significant effect on the residual activation potential that was for acid-treated PLTs: CD62P (MFI = 2770 ± 366), acid-treated and irradiated PLTs:

CD62P (MFI = 2718 ± 266), (Figure 4B), CD63 acid-treated PLTs: MFI = $444 (\pm 25)$ and for CD63 acid treated and irradiated PLTs: MFI = $442 (\pm 30)$ (Figure 5B).

To examine functional defects after acid treatment and irradiation of the PLTs, PS expression was measured on PLTs on Days 1 and 2. Acid treatment and irradiation increased PS expression (Figure S2), but although statistically significant, the difference between untreated and treated PLTs was meager (2.1% in ctr/untreated and 4.5 in acid treated) and the PS expression was not changed significantly from Day 1 to Day 2.

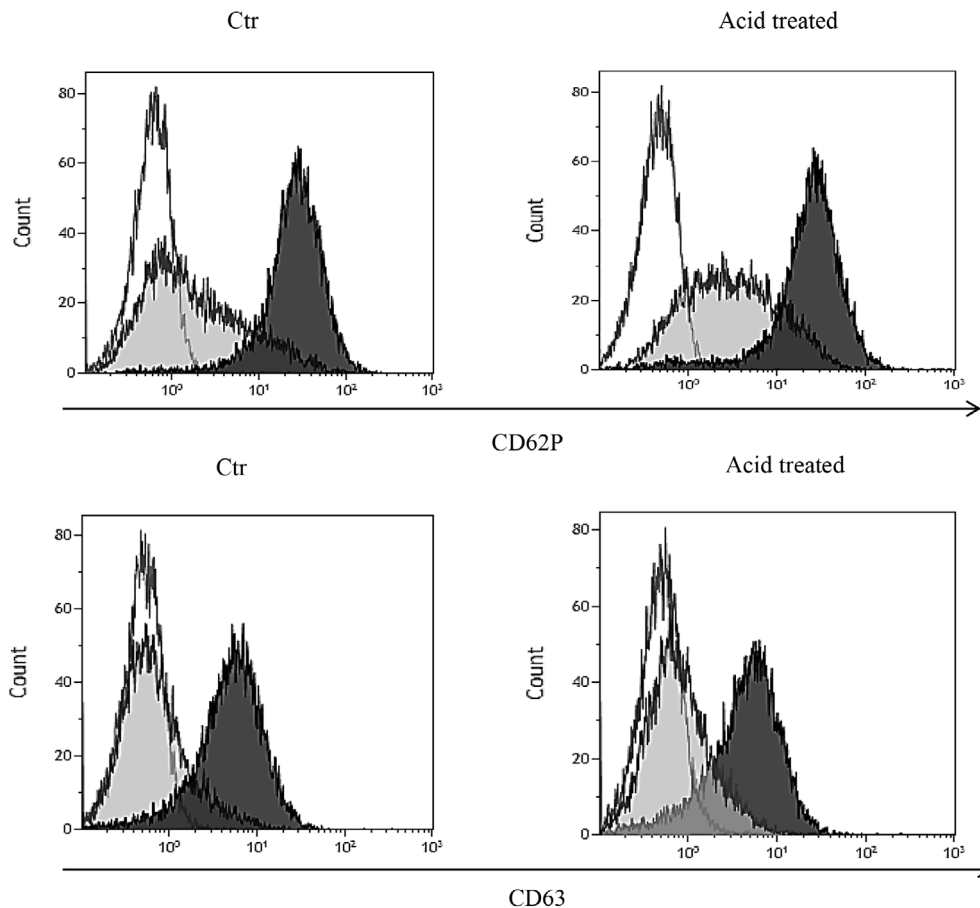


FIGURE 6 Representative histograms for expression of activation markers (CD62P and CD63) were analyzed on untreated (Ctr) and acid-treated PLTs. White peaks represent isotype controls. Gray peaks represent spontaneous activation, (results after 5 minutes on ice \pm acid treatment) and black peaks represent residual activation potential (results after TRAP stimulation)

3.4 | Effect of acid treatment on the function of apheresis PLTs

The PLT functionality was tested by TEG. Acid treatment caused a significant decrease in reaction time for treated PLTs (7.5 minutes \pm 0.6) as compared to untreated PLTs (9 minutes \pm 1.1). However, no significant differences were found in clot kinetics, acid-treated PLTs 1.2 minutes (\pm 0.2), untreated PLTs 1.5 minutes (\pm 0.3), clot strengthening (α), acid-treated PLTs 65.7° (\pm 10.4), untreated PLTs 68.5° (\pm 4.8) or clot strength, PLT function/aggregation (MA), treated PLTs 62.5 mm (\pm 1.3), untreated PLTs 60.3 mm (\pm 2.4). X-ray irradiation did not significantly affect the TEG variables (Figure 7).

3.5 | Acid treatment reduces the binding of anti-HLA to PLTs

The MFI of the untreated PLTs varied from 130 to 30, depending on HLA antibody specificity and degree of incompatibility. Figure 8A shows the results when one single patient sample with multiple anti-HLA Class I antibodies was tested against PLTs from four different

apheresis concentrates, untreated vs acid treated, respectively. It is clear that when antibodies recognize four HLA Class I molecules (HLA-A2, -A24, -B44, and -B62) in PLT Donor 2, we see a high MFI = 131. In contrast, when only one HLA class I molecule was recognized (HLA-A2) in PLT Donor 1, there was a low MFI. In Figure 8B samples from 12 different refractory patients were tested against PLTs from 12 random donors. All samples tested against acid-treated PLTs displayed lower MFIs close to the level of the negative controls.

4 | DISCUSSION

PLT refractoriness remains a clinical challenge associated with an increased risk of bleeding, prolonged hospital stays, and decreased survival. The most common immune causes of PLT refractoriness are antibodies to HLA antigens, especially against HLA Class I. Presence of these antibodies can be caused by prior exposure to pregnancy, transplantation, or transfusions. The current approach to overcome refractoriness caused by anti-HLAs is transfusion with HLA-matched PLTs. An alternative approach is to deplete or denature HLA Class I molecules from PLTs before transfusion. Former studies

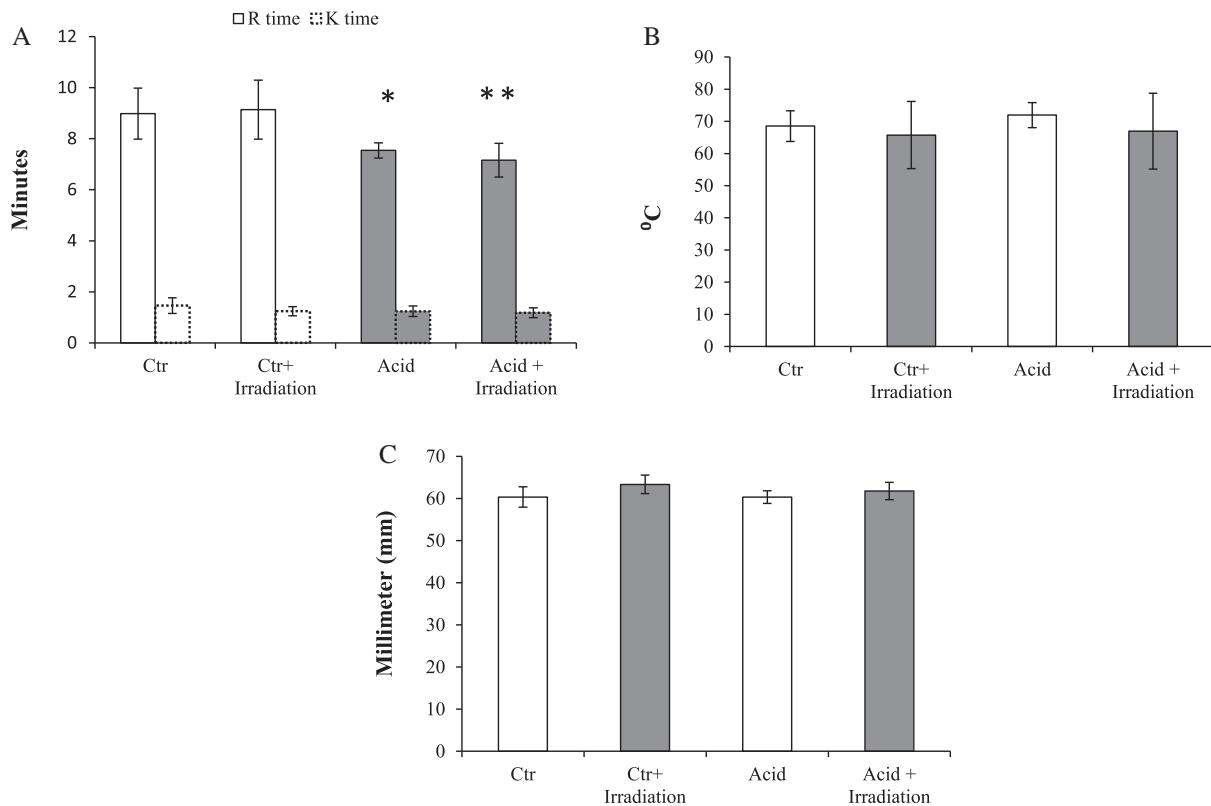


FIGURE 7 Effect of acid treatment and irradiation on TEG variables of apheresis PLTs. The test measures thrombin-activated clotting and was run until the following parameters were defined: (A) R-time = reaction time, (A) K-time = clot kinetics, (B) α angle = steepness of the rising curve, (C) max amplitude (MA) = max distance between the curve lines, reflecting the number and function of the PLTs. Results are given as mean \pm SD ($n = 5$). Student's t test was used to evaluate the statistical significance of the results with P values $< .05$. * P values $< .05$ in comparison with acid-treated and ** in comparison with acid-treated and irradiated PLTs

have demonstrated inconsistent results regarding the efficiency of HLA reduction and PLT viability and function after acid treatment.^{12,24,25} Seven patients have been transfused with acid-treated PLTs, as reported by four different groups. In the patients, acid-treated PLTs gave corrected count increment (CCI) at 1 hour after transfusion comparable to that of HLA-matched PLTs²⁶ and stopped gastrointestinal bleeding.^{27,28} One group did not observe a significant response to two acid-treated PLT concentrates.²⁹ In one patient, a febrile reaction occurred and the observation was stopped 10 minutes after transfusion.²⁵

Recently, it was reported that a short treatment of buffy coat PLT concentrates with citric acid leads to denaturation of the trimolecular HLA Class I complexes without significant damage to the PLTs.¹³ The treatment prevented binding of patient anti-HLA class I antibodies to the PLTs, and antibody-mediated complement activation, and reduced antibody-mediated phagocytosis. However, the functional state of the PLTs after acid treatment should be more closely tested by TEG analysis. The physiological functions of the PLTs remained intact after treatment for at least 4 hours. PLT concentrates can be

obtained by buffy coat-derived pooled PLTs or apheresis and both products are considered equivalent blood components for patient therapy.³⁰ Since previous studies on acid treatment have used PLTs from pooled buffy coats or PLT-rich plasma,^{13,31} it is important also to investigate single apheresis PLTs in this context.

In the current study, we investigated the effect of acid treatment on apheresis-derived PLTs. Acid treatment removed or denatured 70% to 78% of HLA Class I molecules and 74%-80% of β_2 -microglobulin (Figure 1A, and B). Our results are in agreement with the previously reported study by Meinke et al,¹³ which used buffy coat-derived PLTs. The reduction in complement activation demonstrated in the acid-treated PLTs after addition of anti-HLA, seems to correlate with the removal and/or denaturation of HLA antigens on such PLTs. The finding indicates that acid-treated PLTs are protected from HLA antibody-mediated classical complement activation (Figure 2A and B).

Further, our results showed that acid treatment significantly reduced pH value (Figure 3A), which still remained above pH 6.4, the requirement for transfusion in Europe.³² This method shows that adding a 20-fold

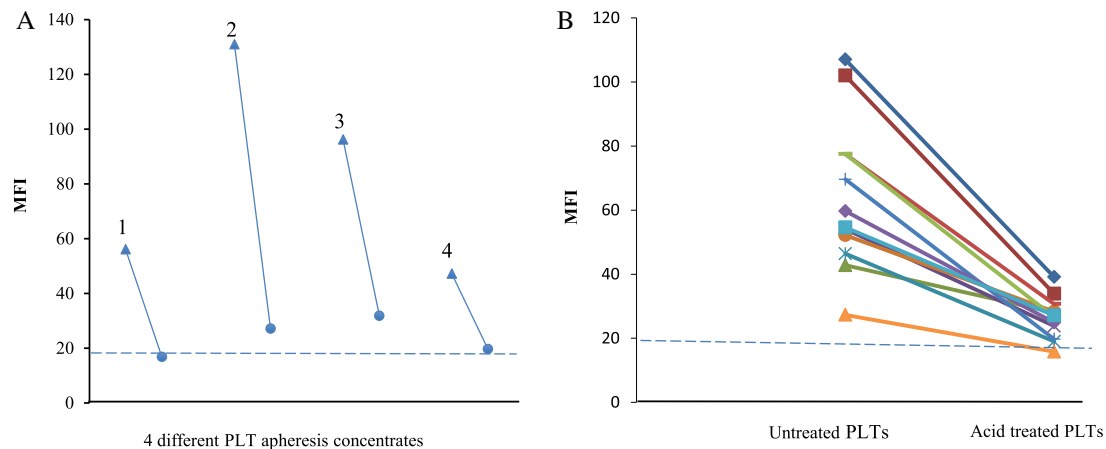


FIGURE 8 MFI of reactive HLA Class I antibodies. Serum from one immunized patient, against 4 untreated (▲) vs acid-treated (●) PLT apheresis concentrates, using flow cytometry. (A) Reactive antibodies against apheresis no. 1: anti-HLA-A2; no. 2: anti-HLA-A2, -A24, -B44, and -B62, no. 3: anti-HLA-A2 and -B57; no. 4: anti-HLA-B44 and -B62. Donor HLA class I genotypes, no. 1: HLA-A2, 3/B7, 51; no. 2: HLA-A2, 24/B62, 44; no. 3: HLA-A2/B7, 57; and no. 4: HLA-A9, 32/B44, 62. Dotted line = negative control. MFI of reactive HLA class I antibodies from 12 refractory patients, against various apheresis concentrates, all untreated vs acid-treated PLTs from the same donors, using flow cytometry. (B) HLA antibody specificities include anti-HLA-A1, -A2, -A3, -A24, -B7, -B8, -B44, -B51, -B57, and -B62 of different titers. Dotted line = negative control

excess volume of ice-cold T-PAS+ after 5 minutes of incubation with citric acid, neutralized the acidic effect of citric acid and restored pH to acceptable values.

We measured the percentage of PLT recovery after acid treatment and after irradiation of acid treated apheresis PLTs. Our results showed that neither acid treatment, which is in line with Meinke et al,¹³ nor irradiation significantly changed the percentage of PLT recovery (Figure 3C). We examined the surface expression of CD62P and CD63 on acid-treated apheresis PLTs to evaluate spontaneous PLT activation. There was a small basic increase in CD62P and CD63 expression on acid-treated PLTs as compared to untreated PLTs (Figures 4A and 5A), which is most likely caused by the exposure to acid, and is in accordance with earlier results.¹³ Further, the residual activation potential of CD62P and CD63 was measured in response to stimulation with TRAP6. These activation markers were strongly upregulated on both untreated and acid treated PLTs (Figures 4B, 5B, and 6). The lack of significant difference between the residual activation potential of acid-treated and untreated PLTs, indicated that the acid-treated PLTs are as efficient as untreated PLTs in initiating and stabilizing hemostasis/coagulation. These results are consistent with those of Meinke et al.¹³ Moreover, the PS expression experiments suggest that functional defects were meager and unchanged over time in the treated PLTs (Figure S2).

In the current study, TEG was used to evaluate the hemostatic function of acid treated PLTs, showing significantly shorter reaction time in acid-treated apheresis

concentrates as compared to untreated PLTs (Figure 7A). R-time reflects the time from the test start (sample placed into the analyzer) until start of clot or fibrin formation. Shorter R-time indicates acceleration of fibrin clot formation. The TEG variable α angle is a reflection of the speed with which clot strength is increasing, and a too low MA indicates a quantitative or functional deficiency of PLTs. Our results demonstrated that α angle and MA values were not significantly different in acid-treated PLTs vs untreated PLTs (Figure 7B and C). It confirms that the quantity and quality of acid-treated PLTs are comparable to untreated PLTs.

When investigating the effect of irradiation on acid-treated apheresis PLTs, we found no significant differences in pH, MPV, activation markers (CD62P, CD63), and TEG variables between X-ray irradiated and nonirradiated PLTs. In accordance with these results, some previous reports^{33–35} found no difference in CD62P and pH between irradiated and nonirradiated PLT concentrates. However, there are also studies that have found differences in pH and CD62P expression between irradiated and nonirradiated PLT concentrates.^{36–39}

We further tested patient samples with multiple anti-HLA Class I antibodies against untreated vs acid-treated PLTs from the same donors. Binding of the patients' anti-HLA Class I reduced markedly, when tested against acid-treated PLTs (Figure 8A and B). Regardless of the number of anti-HLA class I present, the reduction of antibody-binding was efficient in all cases. The results are promising and hopefully transferrable to the in vivo

situation. Our results are in line with the previous study by Meinke et al.¹³

As discussed previously by Meinke et al,¹³ one cannot rule out the possibility that neoantigens, either within denatured HLA Class I antigens or not, could be exposed on the PLT surface after acid treatment. This might render the acid-treated PLTs more immunogenic and give rise to novel alloantibodies that may or may not have a negative impact in recipients. Besides monitoring survival and function of acid-treated PLTs, a clinical study should take this concern into account and if possible specifically monitor presence of neo-alloantibodies.

Our in vitro results suggest that acid-treated and irradiated apheresis PLTs could be used as an alternative to HLA-matched PLT transfusions. Our results open up for a clinical trial, in which acid-treated PLTs in refractory patients can be studied. The relatively simple method of acid stripping could possibly prevent transfusion-associated HLA sensitization and overcome refractoriness. This is especially important in acute situations where no matched PLTs are available but could also save time and costs in prophylactic care compared to HLA-matched donor PLTs. However, before a clinical trial can be initiated, a quantitative proteomics study should be performed to investigate the effect of acid treatment on apheresis PLTs, by identifying possible changes in their proteome. This is the subject of another paper, which we have submitted.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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