blood®
Regular Article

TRANSFUSION MEDICINE

Platelets from donors with consistently low HLA-B8, -B12, or -B35 expression do not undergo antibody-mediated internalization

Anno Saris,[1,2] Bert Tomson,[3] Anneke Brand,[4] Arend Mulder,[4] Frans H. Claas,[4] Jos Lorinser,[5] John Scharenberg,[1,2] S. Marieke van Ham,[1,2] Anja ten Brinke,[1,2] Jaap Jan Zwaginga,[4,6,7,*] and Pieter F. van der Meer[5-7,*]

[1]Department of Immunopathology, Sanquin Research, Amsterdam, The Netherlands; [2]Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands; [3]Department of Transfusion Medicine, Sanquin Blood Bank, Amsterdam, The Netherlands; [4]Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands; [5]Department of Product and Process Development, Sanquin Blood Bank, Amsterdam, The Netherlands; [6]Center for Clinical Transfusion Research, Sanquin Research, Leiden, The Netherlands; and [7]Jon J van Rood Center for Clinical Transfusion Science, Leiden University Medical Center, Leiden, The Netherlands

KEY POINTS

- **HLA-B8, -B12, or -B35 expression on platelets varies significantly between donors and in certain donors is consistently low or undetectable.**
- **Antibody-mediated internalization of platelets correlates with antigen expression and is absent in platelets with low HLA expression.**

Patients refractory to platelet transfusions because of alloimmunization require HLA-matched platelets, which is only possible if a large HLA-typed donor pool is available. However, even then, patients with broad immunization or rare haplotypes may not have suitable donors. In these patients, transfusions with platelets showing low HLA class I expression may be an alternative to fully HLA-matched transfusions. In this study, we quantified the proportion of donors with consistently low HLA-B8, -B12, and -B35 expression on platelets using human monoclonal antibodies specific for these antigens. Furthermore, as model for in vivo clearance, antibody-mediated internalization of these platelets by macrophages was investigated. The expression of HLA-B8, -B12, or -B35 on platelets was extremely variable between individuals (coefficients of variation, 41.4% to 73.6%). For HLA-B8, but not for HLA-B12 or -B35, this variation was in part explained by zygosity. The variation was most pronounced in, but not exclusive to, platelets. Expression within one donor was consistent over time. Remarkably, 32% of 113 HLA-B8, 34% of 98 HLA-B12, and 9% of 66 HLA-B35 donors showed platelet antigen expression that was not or only minimally above background. Antibody-mediated internalization of platelets by macrophages correlated with antibody opsonization and antigen expression and was absent in platelets with low or minimal HLA expression. In conclusion, our findings indicate that a substantial proportion of donors have platelets with consistently low expression of specific HLA class I antigens. These platelets may be used to treat refractory patients with antibodies directed against these particular antigens, despite HLA mismatches. (*Blood*. 2018;131(1):144-152)

Introduction

Platelet (PLT) transfusions are essential for patients with chemotherapy-induced thrombocytopenia. Because of multiple causes, however, 10% to 27% patients can become refractory to PLT transfusion, which is defined as multiple subsequent ineffective transfusions.[1-5] This refractoriness is associated with increased risk for bleeding and reduced survival.[4] Immunological refractoriness occurs in 4% to 8% of all patients receiving PLT transfusions. This is most frequently due to alloimmunization of the recipient against HLAs.[6,7] This alloimmunization results in the production of alloantibodies that target transfused PLTs and induce rapid clearance of the opsonized PLTs by uptake through splenic macrophages.[8-10]

There are currently 2 major strategies for management of patients with PLT refractoriness due to alloimmunization: (1) selecting HLA-identical donors from an HLA-typed donor file and (2) identifying acceptable HLA-mismatched donors by selecting PLTs negative for antigens that are targeted by anti-HLA antibodies from the recipient (ie, antibody specificity avoidance) or by selecting PLTs that are crossmatch negative.[6,11,12] Although these methods result in acceptable posttransfusion increments in 70% to 90% of all cases,[12] both have major limitations. Because the HLA system is highly polymorphic, a very large donor pool is needed, and for patients with multiple antibody specificities and/or rare HLA phenotypes, which occurs more frequently with increasing immigration, sufficiently compatible donors might not be available. An improved or alternative donor-selection strategy, as compared with fully HLA-matched PLTs products, would therefore be welcome to treat refractory patients.

A lower antigen expression may render PLTs less susceptible to antibody-mediated clearance by macrophages.[8,13-15] Therefore, strategies to minimize or reduce PLT HLA class I expression were explored as a means to avoid HLA matching to treat refractory patients. In this regard, using chloroquine phosphate isotonic fluid or citric acid HLA class I from the PLT surface was denaturated, causing a 70% to 80% loss of serologic activity.[16-19] Clinical application of these PLTs, however, resulted in varying outcomes, presumably due to manipulation-induced PLT function defects.[20] An alternative strategy for HLA class I elution may be to use donors that have naturally low expression of HLA class I on their PLTs, as has been described for certain HLA-B antigens. Indeed, a study that transfused HLA-B12–mismatched PLTs to refractory patients because of alloimmunization showed satisfactory increments in 69% of all transfusions.[21] In this study, however, the density of HLA-B12 expression on the transfused PLTs was not determined, which might explain why some patients failed to show satisfactory increments. From all HLA-B antigens, the most frequent alleles in the Dutch donor population are HLA-B7 (27.3%), -B12 (25.3%), -B8 (22.7%), and -B35 (17.8%).[22] High variation of HLA-B8, -B12, and its split antigen B44 expression on PLTs has been reported, whereas this variation was not observed in HLA-B7.[23-26] Identifying donors with naturally low expression of frequent class I antigens on PLTs could significantly improve the availability of HLA-matched donors for PLT supportive care of immunized patients.

We investigated whether low HLA class I–expressing PLTs might overcome antibody-mediated clearance of transfused PLTs despite HLA mismatches and in this respect be an alternative strategy for fully HLA-matched transfusions. To do this, we first established the proportion of donors with consistently low expression of HLA-B8, -B12, and -B35 and then studied the effect of this low HLA expression on antibody-mediated internalization of PLTs by macrophages, which was shown to correlate with in vivo clearance.[27]

Materials and methods

Samples

To determine specific HLA class I expression and internalization of PLTs with high or low expression, citrated blood samples from HLA-typed donors were obtained after blood donation. Blood from these donors was collected multiple times, with a maximum of 10 samples per donor. HLA typing was performed in different HLA laboratories in The Netherlands as routine blood bank practice for PLT donors using low-, intermediate-, or high-resolution DNA or split-level serologic typing. Furthermore, to isolate monocytes or determine internalization of differently opsonized PLTs, blood samples from anonymous healthy individuals were obtained after they gave written informed consent. This study was approved by the Sanquin Ethical Advisory Board in accordance with the Declaration of Helsinki and according to Dutch regulations.

Allele-specific HLA class I antibody production

To specifically determine HLA-B8, -B12, and -B35 expression on PLTs, human monoclonal antibodies (mAbs) were selected from a locally produced mAb panel, produced in stirred-tank format and purified as previously described.[28,29] mAbs were labeled according to the manufacturer's instructions using an Alexa Fluor 488 fluorescent labeling kit (Molecular Probes/Invitrogen, Breda, The Netherlands). Labeling and staining efficiency was standardized by flow cytometry analysis as previously described.[28] In short, after fluorescent labeling, a new lot of antibodies was compared with the previous lot using HLA-typed peripheral blood mononuclear cells. The dilution of the new lot was adjusted to achieve equal fluorescent signals for the new and the old batch. Staining was performed in saturating concentrations.

Donor HLA class I expression

To determine specific HLA class I expression on PLTs, blood was diluted to 2.5×10^6 PLTs/mL with PBS supplemented with 8 mM EDTA (Sigma, Zwijndrecht, The Netherlands). PLTs were stained 30 min at room temperature (RT) using 50 μg/mL anti-HLA-B8 (clone BVK1F9), anti-HLA-B12 (clone JOK3H5 combined with clone DK7C11) or anti-HLA-B35 (clone HDG8D9) Alexa Fluor 488–labeled antibodies and anti-CD61 labeled with PE-Cy7 (Beckman Coulter, Woerden, The Netherlands). Specific HLA class I expression on white blood cells (WBCs) from the same individuals was determined in whole blood supplemented with 8 mM EDTA and 50 μg/mL anti-HLA-B8, anti-HLA-B12, or anti-HLA-B35 Alexa Fluor 488–labeled mAbs and anti-CD45 labeled with PE-Cy5 (Beckman Coulter). After 30-min incubation at RT, red cells were lysed using IO Lysing Solution according to the manufacturer's instructions (Beckman Coulter). Subsequently, samples were measured using flow cytometry (Cytomic FC500 MPL flow cytometry system with MXP software, Beckman Coulter) and analyzed with Kaluza Flow Cytometry Analysis Software (V1.3, Beckman Coulter). The gating strategy involved both selection of single PLTs or lymphocytes (based on forward and side scatter), and CD61-positivity for PLTs or CD45 positivity for WBCs (supplemental Figure 1, available on the *Blood* Web site). To determine background fluorescence levels, during each experiment, a negative control (ie, blood from a donor negative for the specified HLA type) was measured (n = 112, 90, and 76 for HLA-B8, -B12, and -B35). Specific HLA expression was presented relative to the averaged background median fluorescence as mean ± standard deviation (SD). Coefficients of variation were calculated by dividing the SD with the mean, multiplied by 100%.

PLT isolation and opsonization

Citrated blood from healthy individuals or donors with known low (ie, <2 SDs above background) or high (ie, above median) specific HLA class I expression was collected, and PLT-rich plasma was obtained by centrifugation at 200 radial centrifugal force for 10 min. PLT-rich plasma was washed and labeled for 20 min in PBS at RT with 3.75 μM PKH26 membrane dye (Sigma) with continuous gentle shaking. Subsequently, PLTs from healthy individuals were opsonized with anti-HPA1 AMAb (humanized IgG1, clone B2G1; kindly provided by G. Vidarsson, Sanquin Research, Amsterdam, The Netherlands) or anti-pan HLA class I mAb (mouse IgG2a, clone W6-32; ITK Diagnostics, Uithoorn, The Netherlands), and PLTs from donors with the specific HLA antigens were opsonized with 50 μg/mL anti-HLA-B8, anti-HLA-B12, or anti-HLA-B35 human IgG1 mAbs. After 30-min opsonization at RT, PLTs were washed twice using sequestrine buffer (17.5 mM Na_2HPO_4, 8.9 mM Na_2EDTA, 154 mM NaCl, pH 6.9, containing 0.1% [wt/vol] bovine serum albumin [BSA]; all obtained from Merck Millipore, Amsterdam, The Netherlands).

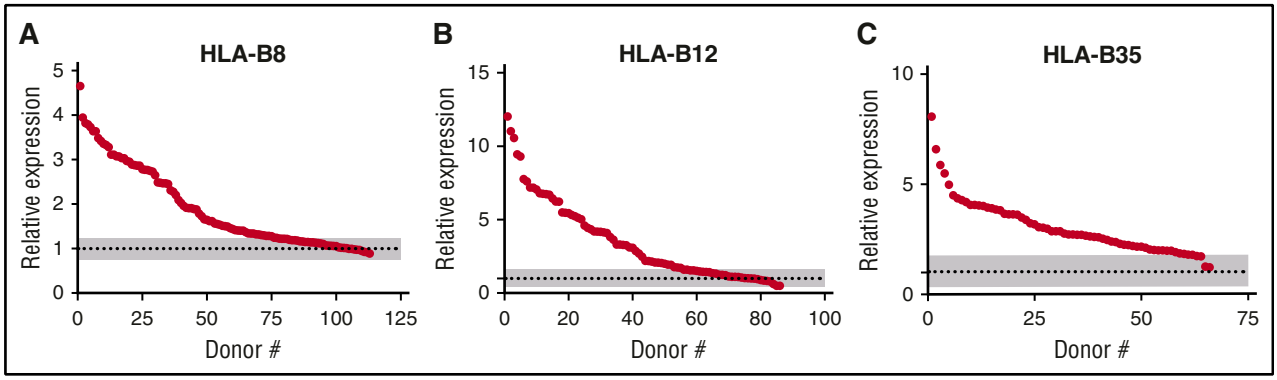


Figure 1. Expression of HLA-B8, HLA-B12, and HLA-B35 on PLTs varies significantly between individuals. Specific HLA class I expression on PLTs was determined using Alexa Flour 488–labeled mAbs that specifically target HLA-B8 (A), HLA-B12 (B), or HLA-B35 (C). For each donor, relative median fluorescence intensities were averaged and are represented by 1 circle. Donors were sorted based on their expression levels. Staining from donors negative for the specific HLA allele is depicted by a dotted line (mean) and gray area (mean ± 2 SDs).

Internalization assay

Monocytes were isolated from fresh apheresis material using the Elutra Cell Separation System (Gambro, Lakewood, CO) and subsequently frozen until further use. The >90% purity of these monocytes was confirmed using flow cytometry. On day 0, monocytes were thawed and differentiated in a 6-well plate (Nunc/Sanbio BV, Uden, The Netherlands) to macrophages with 10 ng/mL granulocyte-macrophage colony-stimulating factor (Cellgenix, Freiburg, Germany) in Iscove modified Dulbecco medium (Lonza, Breda, The Netherlands) supplemented with 10% fetal calf serum (Bodinco, Alkmaar, The Netherlands), 100 U/mL penicillin, and 100 U/mL streptomycin (both from Gibco/Thermo Fischer Scientific, The Netherlands) at 37°C and 5% CO_2. On days 7 to 9, macrophages were incubated for 60 min at 37°C with opsonized PLTs. Subsequently, macrophages were washed with PBS + 0.5% BSA, harvested using 130 mM lidocaine (Sigma) with 10 mM EDTA (Merck Millipore), washed again in PBS, fixed with 3.7% paraformaldehyde (Sigma) in PBS, washed 2 more times in PBS + 0.5% BSA, and incubated with FITC-labeled anti-CD61 (Beckman Coulter) and APC or brilliant violet 650-labeled anti-HLA DR (both from BD biosciences, Breda, The Netherlands). After 30 min incubation at RT, cells were washed and analyzed using imaging flow cytometry (ImageStreamX Mark II Imaging Flow Cytometer, Merck Millipore). Samples were analyzed using IDEAS Application (IDEAS software V6.1.303.0, Merck Millipore). The gating strategy is presented in supplemental Figure 2 and involved selection of single macrophages (aspect ratio intensity vs area; for bright field and APC/BV650 channel) in focus (gradient root mean square; for bright field and APC/BV650 channel) and exclusion of false-positive cells (defined as intracellular fluorescence of the PLT-specific anti-CD61 membrane staining). PLT internalization by macrophages was determined by quantifying the amount of PKH fluorescence within macrophages (using fluorescence intensity within an intracellular mask based on HLA DR membrane staining). To correct for varying "baseline" internalization of PLTs (presumably caused by differences in quality and age [1 to 3 days] due to transportation), internalization of HLA-typed opsonized PLTs was presented relative to internalization of unopsonized PLTs from the same donor.

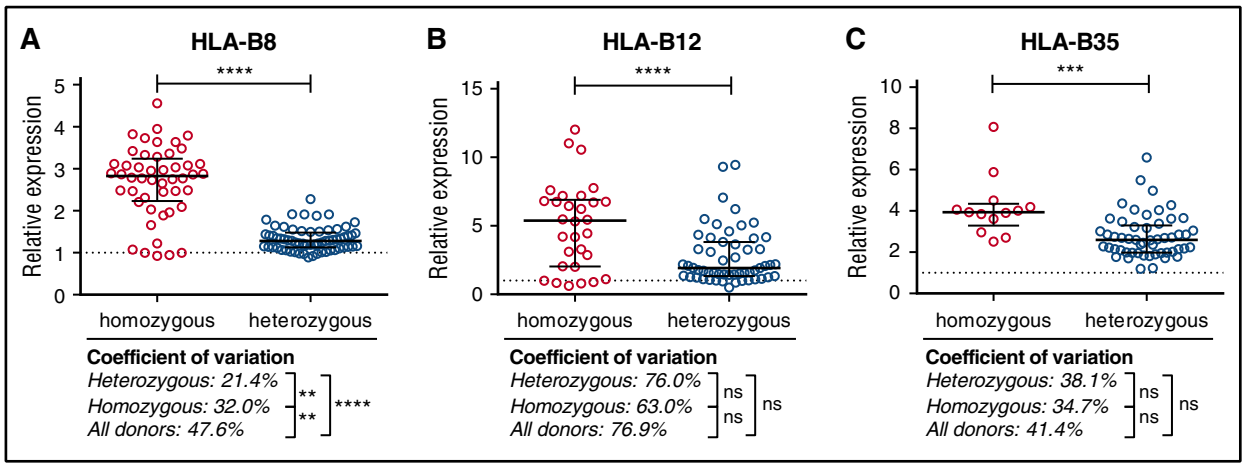


Figure 2. Interindividual variation in HLA-B8, B12, and B35 expression on PLTs is only partially explained by homozygous or heterozygous haplotypes. HLA-B8– (A), -B12– (B), and -B35–positive (C) donors were divided in 2 groups based on zygosity. Relative expression is presented as median ± interquartile range, and each donor is represented by one symbol. Background staining is depicted with the dotted line. Expression between heterozygous and homozygous donors was compared with an unpaired Student *t* test, and a F-test was used to investigate differences in variation between groups. Statistical significance is depicted as $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$; ns, not significant.

Statistical analysis

Statistical analysis and graphical presentation was performed using GraphPad Prism version 7.02 (GraphPad Software, La Jolla, CA). The percentage of false-negative test results (ie, <2 SDs above background) was determined using a sensitivity analysis in donors with high HLA class I expression on average, defined as >2 SDs above background, with at least 3 measurements. Correlations between HLA class I expression in WBCs and PLTs as well as PLT opsonization and internalization were calculated using a Pearson's correlation test. To investigate the effect of zygosity on interindividual variation, expression data were divided based on zygosity and then normalized to the group average (ie, all donors, homozygous or heterozygous), and subsequently, an F-test was used to calculate statistical differences in variation between these groups. Statistical differences between interindividual and intraindividual variation in HLA class I expression were calculated using an F-test. Statistical differences in HLA expression were calculated using an unpaired Student *t* test. Statistical differences in internalization due to opsonization were calculated using a Wilcoxon matched-paired signed rank test, and differences in internalization between positive controls from PLTs with high or low expression were determined by Mann-Whitney *U* test. $P < .05$ was considered statistically significant.

Results

HLA class I expression: variation between blood donors

PLTs and WBCs from HLA-B8– (n = 113), -B12– (n = 86), and -B35–positive donors (n = 66) were analyzed for the level of these specific HLA-B antigens. From donors positive for the broad HLA-B12 antigen, most donors were positive for the split antigen B44 (n = 85), and 6 donors were B45 positive. Because only 1 of these 6 B45-positive donors was B44 negative, potential differences between the HLA-B12 split antigens were not studied. All expression data are presented relative to the background values of donors negative for the specific antigen as relative median fluorescence intensities. PLTs from positive individuals showed a relative HLA-B8, -B12, and -B35 expression of 1.9 ± 0.9, 3.2 ± 2.6, and 3.1 ± 1.3 (mean ± SD), respectively (Figure 1), with a maximum variation between individuals of approximately fivefold, 12-fold, and eightfold, respectively. Even more interesting is that from all HLA-B8, -B12, and -B35 donors, 31.9%, 33.7%, and 9.1%, respectively, showed no or only minimal expression (defined as <2 SDs above background levels) of these HLA class I antigens on PLTs.

HLA class I expression: causes of the observed variation

Next, we investigated the effect of zygosity on specific HLA class I expression. For this, donors were grouped as heterozygous or homozygous for a given HLA-B antigen, and the effect of this grouping on interindividual variation was investigated using an F-test. PLTs from homozygous HLA-B8 donors had significantly higher expression (2.7 ± 0.9, n = 48) than heterozygous B8 donors (1.3 ± 0.3, n = 65) (Figure 2A). This increased expression in homozygous individuals was also observed for HLA-B12 (5.0 ± 3.2 [n = 30] vs 2.8 ± 2.1 [n = 56]) (Figure 2B) and -B35 (4.2 ± 1.5 [n = 13] vs 2.8 ± 1.0 [n = 53]) donors (Figure 2C). In the group of homozygous HLA-B8, -B12, and -B35 donors, 12.5% (6 of 48 donors), 20.0% (6 of 30 donors), and 0.0% (0 of 13 donors), respectively, showed expression that was not or only minimally detectable. Furthermore, grouping HLA-B8 donors based on their zygosity significantly reduced interindividual variation in expression, wherein it decreased from homo- to heterozygous donors. However, in HLA-B12– and -B35–positive donors, although the density of expression was affected by zygosity, the interdonor variation was not. To investigate whether low antigen

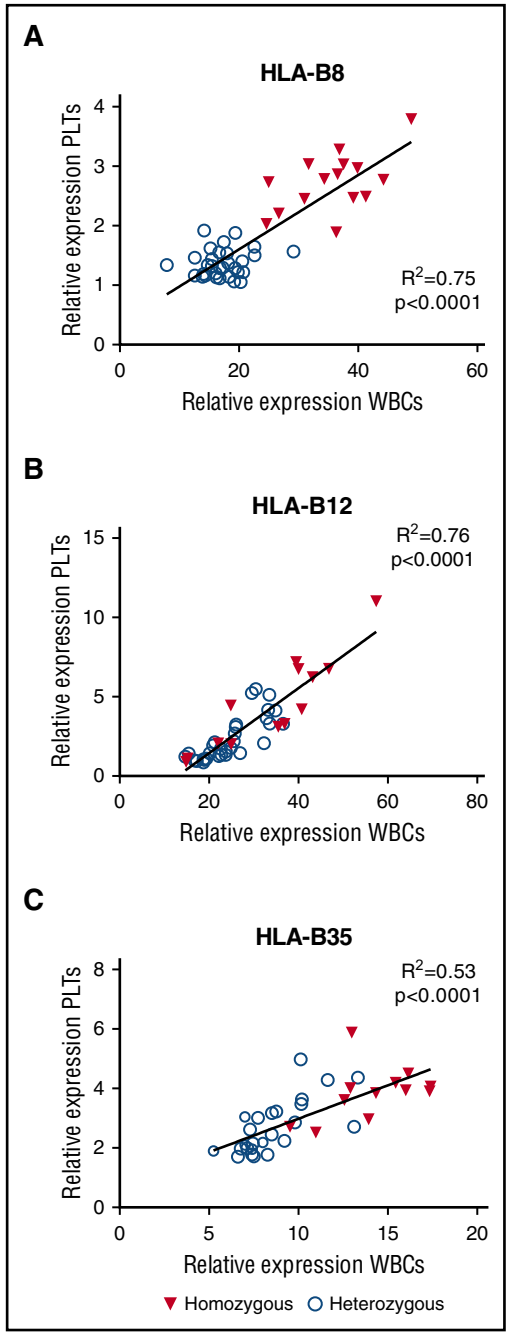


Figure 3. PLT HLA-B8, -B12, and -B35 expression correlates with specific HLA expression on WBCs. Specific HLA class I expression of PLTs was correlated to the expression of WBCs obtained from the same individual (HLA-B8 [A], -B12 [B], and -B35 [C]). PLT and WBC relative median fluorescence intensities were averaged for each donor and are represented by one symbol. A Pearson correlation test was performed to determine the association between specific HLA class I expression on PLTs and WBCs from the same individual.

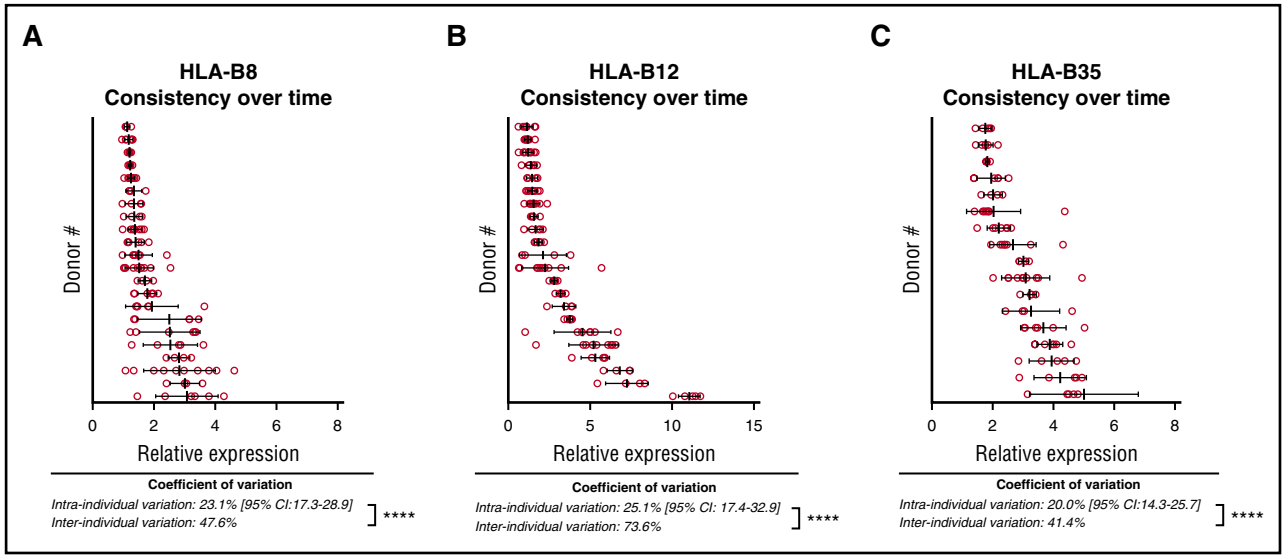


Figure 4. PLT HLA class I expression of each donor is constant over time. For each donor with at least 4 measurements, specific HLA I expression is depicted for HLA-B8 (A), -B12 (B), and -B35 (C). For each donor, relative expression is presented as mean ± SD, and each measurement is depicted by one symbol. Differences between interindividual and intraindividual variation were calculated using an F-test. Statistical significance is depicted as ****$P < .0001$.

expression is a general donor characteristic, individuals who expressed two of the investigated antigens (ie, HLA-B8/B12, -B8/B35, or -B12/B35) were selected and the association of expression of these 2 antigens was investigated. Expression of these HLA-B antigens was not correlated, but the number of donors expressing 2 of the investigated antigens was limited (n = 6-8). However, donors with low expression of both B antigens were identified (supplemental Figure 3). To investigate whether the observed variations are exclusive to PLTs, specific HLA class I expression was also studied on WBCs from the same individual. HLA-B8, -B12, and -B35 expression on WBCs similarly varied between individuals and again only for HLA-B8 donors due to zygosity (Figure 3). The expression on WBCs clearly correlated with the observed variability of these antigens on PLTs, although expression on WBC always was strongly above the background level, showing that the observed variations are not PLT specific.

HLA class I expression: consistency over time

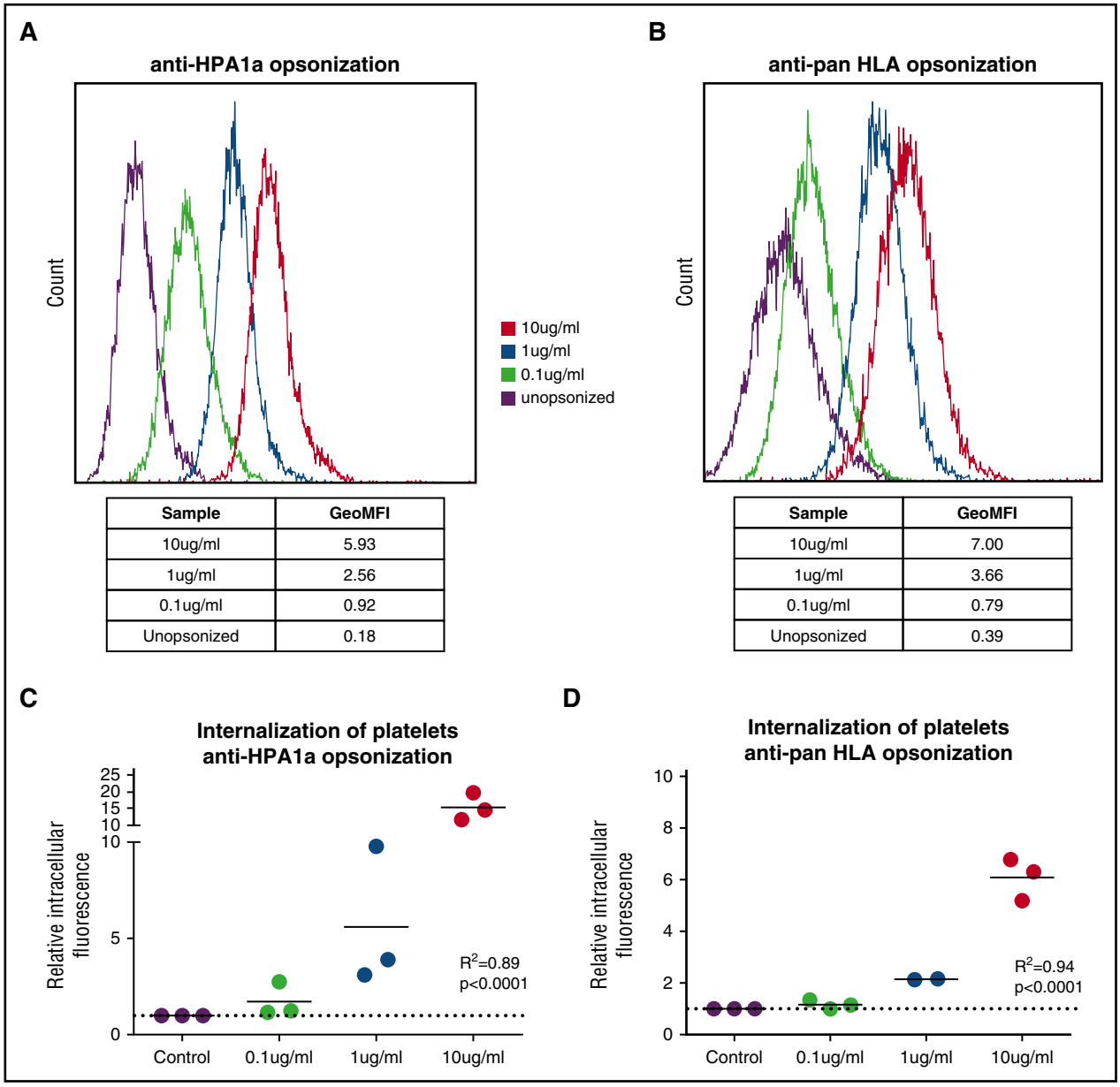
To investigate the consistency of expression on PLTs within an individual (ie, intraindividual variation), donors with at least 4 independent blood samplings at different time points were selected (Figure 4). The average follow-up time was 349, 413, and 371 days for HLA-B8, -B12, and -B35 donors, respectively. The variation in HLA-B8 (n = 22), -B12 (n = 22), and -B35 (n = 17) expression within each individual donor was significantly lower than the variation between individuals (Figure 4A-C), which was most pronounced in low-expressing donors. Because of these low intraindividual variations, individuals with consistently low expression can be used as possible HLA-mismatched PLT donors. However, some donors with a high expression on average may show a lower expression in another sample, which may affect the firm definition of a donor with consistently low expression if only 1 sample is tested. A sensitivity analysis (using a threshold of 2 SDs above background median fluorescence intensity defining low expression) showed that the possibility of a false-negative test result was 20.3%, 8.4%, and 7.6% for HLA-B8–, -B12–, and -B35–positive donors. As a result, 2 consecutive negative test results indicate with >95% certainty the identification of a donor with low HLA expression.

Effect of antigen expression on antibody-mediated internalization

Next, the effect of reduced antigen expression on antibody-mediated internalization of PLTs by macrophages was studied. First, PLTs were incubated with increasing concentrations of mAbs (0.1, 1, and 10 μg/mL) that target either HPA1a or pan-HLA class I, resulting in increased opsonization (Figure 5A-B). The opsonization of PLTs led to antibody-mediated internalization by macrophages (Figure 5C-D), which strongly correlated with the level of antibody opsonization ($r^2 = 0.89$ for anti-HPA1a and $r^2 = 0.94$ for anti-pan HLA class I). To subsequently determine the relevance of the observed variations in HLA class I expression, PLTs from donors with high (ie, expression above median) or low (ie, <2 SD above background) HLA-B8, -B12, or -B35 expression were obtained and opsonized with the relevant human mAbs in saturating concentrations. Compared with unopsonized controls, internalization of PLTs obtained from donors with high specific HLA class I expression significantly increased due to opsonization, which was not observed for PLTs obtained from donors with low specific HLA class I expression (Figure 6A). PLT opsonization with an irrelevant antibody (ie, anti-HPA1a) did not result in differences in internalization of PLTs obtained from high- or low-expressing donors (Figure 6B). Antibody-mediated internalization of PLTs correlated with PLT antigen expression (Figure 6C).

Discussion

HLA-matched PLT transfusions require the maintenance of a large HLA-typed donor pool. The efficiency of this HLA matching could be significantly improved if certain mismatches are allowed due to low or undetectable antigen expression. In this study, we explored the proportion of donors with naturally low expression of HLA-B8, -B12, or -B35 on PLTs. Second, the



Sample	GeoMFI
10ug/ml	5.93
1ug/ml	2.56
0.1ug/ml	0.92
Unopsonized	0.18

Sample	GeoMFI
10ug/ml	7.00
1ug/ml	3.66
0.1ug/ml	0.79
Unopsonized	0.39

Figure 5. The degree of antibody opsonization correlates with the degree of antibody-mediated internalization of PLTs by macrophages. PLTs from healthy individuals were incubated with 2 antibodies (anti-HPA1a [A,C] and anti-pan HLA class I [B,D]) in increasing concentrations (0.1 μg/mL, 1 μg/mL, and 10 μg/mL). As negative controls, PLTs were left unopsonized. Representative plots of 3 individual experiments are depicted (A-B). PLTs were incubated with macrophages, and PLT internalization was quantified. (C-D). A Pearson correlation test was used to determine the association between PLT antibody opsonisation and PLT internalization by macrophages.

consistency of this expression was determined. Finally, the effect of this low expression on antibody-mediated internalization of PLTs by macrophages was investigated. Interindividual variation in HLA-B8, -B12, and -B35 expression on PLTs was high, with a maximum variation between individuals of approximately fivefold, 12-fold, and eightfold, respectively. The observed variations in the present study for HLA-B8 and -B12 were less than the previously reported eightfold and 35-fold variations.[23,24] This difference is probably because we used flow cytometry assay in our study, whereas a 2-stage ^{51}Cr release assay was used previously.

Although the variation in specific HLA class I expression was most prominent on PLTs, expression also varied on WBCs. As PLT and WBC antigen expression appeared correlated, the observed interdonor variations are not PLT but donor specific and suggest involvement of (epi-)genetic factors. For HLA-B8, the inter-individual variations are indeed in part explained by zygosity. In addition, low expression of one HLA-B antigen was not associated with low expression of another B-antigen, possibly indicating that the density of antigen expression is dependent on the allele. However, although posttranslational modifications are known to affect surface expression of HLA-A, HLA-B, and HLA-C and thereby cause variations between HLA alleles,[30,31] this does not explain variations within one HLA allele. Furthermore, inflammation causes significant upregulation of mainly HLA-B on WBCs,[32] which is less likely to play a role in our studies, which only involved healthy donors. Clearly, more

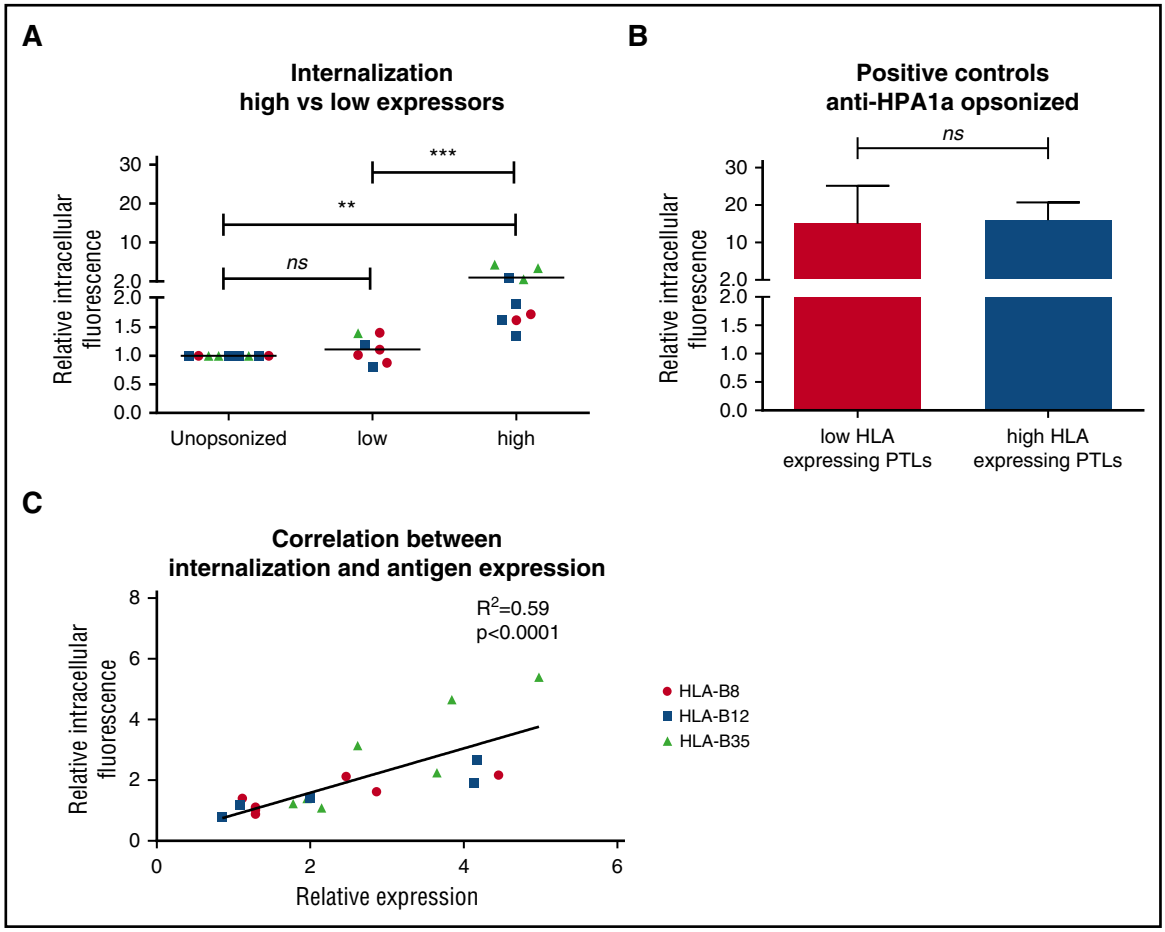


Figure 6. Opsonization of PLTs with low HLA-B8, -B12, or -B35 expression does not lead to antibody-mediated internalization by macrophages. PLTs with low (n = 7), intermediate (n = 4), or high (n = 9) HLA-B8, -B12, or -B35 expression were left unopsonized or opsonized with anti-HLA-B8, anti-HLA-B12, or anti-HLA-B35, and internalization by macrophages was determined (A). Positive controls were opsonized with anti-HPA1a (B). Antibody-mediated internalization was correlated with antigen expression using a Pearson correlation test (C). Intracellular fluorescence was normalized against unopsonized PLTs from the same individual and presented as mean (A,C) or mean ± SEM (B). Statistical differences in internalization were determined using a Wilcoxon matched-pair test (A, unopsonized vs low/high) or Mann-Whitney *U* test (A, high vs low; B). Statistical significance is depicted as $^{**}P < .01$, $^{***}P < .001$.

studies are warranted to further explore the underlying causes of these interindividual differences in HLA class I expression.

In contrast to the high interindividual variation, specific HLA class I expression appeared to be fairly constant within each individual, particularly in the low-expressing individuals. This allows identification of donors with consistently low HLA-B8, -B12 or -B35 expression. Because of this consistency, instead of testing each PLT concentrate for its level of HLA expression, donors can be marked in the HLA-typed donor file as "low expressor" for the specific HLA allele. This, in combination with a straightforward flow cytometry–based assay, allows simple clinical implementation. However, incidental low expression in donors with on average high expression requires retesting of each donor displaying low expression on the first measurement. Whatever the underlying reason for this, 2 consecutive measurements showing a low expression identifies a low-expressing donor with >95% certainty.

PLT concentrates with a naturally low level of specific HLA class I expression obviously have major advantages over the chemical treatment of PLTs to lower HLA expression. Although acid treatment results in a 70% to 80% loss of serological activity, the clinical effectiveness is disappointing. This has been attributed to manipulation-induced PLT dysfunction, thereby limiting clinical application.[16,18,20] The use of unmanipulated naturally low HLA class I expression on PLTs, which is present in 31.9%, 33.3%, and 9.1% of HLA-B8–, -B12–, and -B35–positive donors when using a cutoff <2 SDs above background, would not have this disadvantage. Homozygous donors with such low expression on PLTs, as observed for HLA-B8 and -B12, are of particular interest, because these only need to be matched for HLA-A, thereby greatly increasing the possibility of matching a patient with a donor.

For transfusion purposes, antigen expression on PLTs must be reduced to such an extent that anti-HLA class I antibody–mediated clearance of PLTs by macrophages is limited or completely abolished.[9,10] Because Lim and colleagues have previously shown that antibody-mediated internalization in vitro correlates with in vivo clearance,[27] our data showing an absence of antibody-mediated internalization of low-HLA-expressing PLTs by macrophages are of particular interest. Because this antibody-mediated internalization showed a linear correlation with the degree of antibody opsonization and PLT antigen

expression, it is likely that PLT antigen expression will also correlate with clearance in vivo. However, the degree of antibody opsonization depends not only on antigen expression but also on antibody titer, avidity, and/or isotype. Therefore, the specific threshold below which HLA class I expression will not result in antibody-mediated clearance will be patient specific and can not be predicted at this point.

A limitation of this study is the lack of clinical data supporting the findings observed in our phagocytosis experiment, even though this experimental setup was proven to have clinical correlation.[27] If PLT transfusions with low expressing mismatched HLA-B alleles actually result in satisfactory increments in broadly immunized and refractory patients could be evaluated in a parallel-group semirandomized trial,[33] in which patients with immunological refractoriness should be randomized to receive either PLTs with high or low HLA-B8, -B12, or -B35 expression.

In summary, we show high interindividual variation for HLA-B8, -B12, and -B35 expression on PLTs, with 9% to 34% of donors showing reproducibly undetectable or low expression. PLTs with low HLA expression showed deficient antibody-mediated internalization by macrophages. Although such low HLA expression on PLTs is mostly found in heterozygous donors, even homozygous HLA-B8 and -B12 individuals may show expression at background levels. In theory, for such donors, only HLA-A needs to be taken into account as matching criteria, which would greatly facilitate donor selection for alloimmunized patients. Establishing a pool of donors with such consistently low expression of HLA-B antigens might even allow transfusions of highly immunized patients during acute situations. Future studies should explore clinical effectiveness of such a "low HLA expressed mismatch" strategy and determine which expression levels result in satisfactory increments in refractory patients due to alloimmunization.

Acknowledgments

The authors thank Susan Cuvalay (Sanquin Blood Bank) for her help with the transportation of study material, all individuals for giving informed consent, and G. Vidarsson for kindly providing the anti-HPA1a mAb.

This study was funded by Product and Process Development Cellular products (Sanquin PPOC) 09-026.

Authorship

Contribution: A.S. designed research, performed experiments, analyzed and interpreted data, and wrote the manuscript; B.T. and A.B. designed research and critically reviewed manuscript; A.M. and F.H.C. developed mAbs and reviewed the manuscript; J.L. and J.S. performed experiments; S.M.v.H. interpreted data and reviewed the manuscript; and A.t.B., J.J.Z., and P.F.v.d.M designed research, interpreted data, and critically reviewed the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Pieter F. van der Meer, Plesmanlaan 125, 1066CX Amsterdam, The Netherlands; e-mail: p.vandermeer@sanquin.nl.

Footnotes

Submitted 28 July 2017; accepted 20 October 2017. Prepublished online as *Blood* First Edition paper, 1 November 2017; DOI 10.1182/blood-2017-07-799270.

*J.J.Z. and P.F.v.d.M. contributed equally to this study.

The online version of this article contains a data supplement.

There is a *Blood* Commentary on this article in this issue.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

REFERENCES

1. Seftel MD, Growe GH, Petraszko T, et al. Universal prestorage leukoreduction in Canada decreases platelet alloimmunization and refractoriness. *Blood*. 2004;103(1):333-339.
2. Bilgin YM, van de Watering LMG, Brand A. Clinical effects of leucoreduction of blood transfusions. *Neth J Med*. 2011;69(10):441-450.
3. Trial to Reduce Alloimmunization to Platelets Study Group. Leukocyte reduction and ultraviolet B irradiation of platelets to prevent alloimmunization and refractoriness to platelet transfusions. *N Engl J Med*. 1997;337(26):1861-1869.
4. Kerkhoffs JLH, Eikenboom JCJ, van de Watering LMG, van Wordragen-Vlaswinkel RJ, Wijermans PW, Brand A. The clinical impact of platelet refractoriness: correlation with bleeding and survival. *Transfusion*. 2008;48(9):1959-1965.
5. Slichter SJ, Davis K, Enright H, et al. Factors affecting posttransfusion platelet increments, platelet refractoriness, and platelet transfusion intervals in thrombocytopenic patients. *Blood*. 2005;105(10):4106-4114.
6. Stanworth SJ, Navarrete C, Estcourt L, Marsh J. Platelet refractoriness–practical approaches and ongoing dilemmas in patient management. *Br J Haematol*. 2015;171(3):297-305.
7. Pavenski K, Freedman J, Semple JW. HLA alloimmunization against platelet transfusions: pathophysiology, significance, prevention and management. *Tissue Antigens*. 2012;79(4):237-245.
8. Badlou BA, Wu YP, Smid WM, Akkerman J-WN. Platelet binding and phagocytosis by macrophages. *Transfusion*. 2006;46(8):1432-1443.
9. Grozovsky R, Hoffmeister KM, Falet H. Novel clearance mechanisms of platelets. *Curr Opin Hematol*. 2010;17(6):585-589.
10. Crow AR, Lazarus AH. Role of Fcgamma receptors in the pathogenesis and treatment of idiopathic thrombocytopenic purpura. *J Pediatr Hematol Oncol*. 2003;25(suppl 1):14-18.
11. Rebulla P. A mini-review on platelet refractoriness. *Haematologica*. 2005;90(2):247-253.
12. Engelfriet CP, Reesink HW, Aster RH, et al. Management of alloimmunized, refractory patients in need of platelet transfusions. *Vox Sang*. 1997;73(3):191-198.
13. Lux A, Yu X, Scanlan CN, Nimmerjahn F. Impact of immune complex size and glycosylation on IgG binding to human FcγRs. *J Immunol*. 2013;190(8):4315-4323.
14. Leidi M, Gotti E, Bologna L, et al. M2 macrophages phagocytose rituximab-opsonized leukemic targets more efficiently than m1 cells in vitro. *J Immunol*. 2009;182(7):4415-4422.
15. Koval M, Preiter K, Adles C, Stahl PD, Steinberg TH. Size of IgG-opsonized particles determines macrophage response during internalization. *Exp Cell Res*. 1998;242(1):265-273.
16. Kao KJ. Selective elution of HLA antigens and beta 2-microglobulin from human platelets by chloroquine diphosphate. *Transfusion*. 1988;28(1):14-17.
17. Kurata Y, Oshida M, Take H, et al. New approach to eliminate HLA class I antigens from platelet surface without cell damage: acid treatment at pH 3.0. *Vox Sang*. 1989;57(3):199-204.
18. Novotny VM, Doxiadis IN, van Doorn R, Brand A. The kinetics of HLA class I elution and the relevance for the use of HLA-eluted platelet transfusions. *Br J Haematol*. 1996;95(2):416-422.

19. Meinke S, Sandgren P, Mörtberg A, et al. Platelets made HLA deficient by acid treatment aggregate normally and escape destruction by complement and phagocytes in the presence of HLA antibodies. *Transfusion*. 2016;56(2):370-382, quiz 369.

20. Novotný VM, Doxiadis II, Brand A. The reduction of HLA class I expression on platelets: a potential approach in the management of HLA-alloimmunized refractory patients. *Transfus Med Rev*. 1999;13(2):95-105.

21. Schiffer CA, O'Connell B, Lee EJ. Platelet transfusion therapy for alloimmunized patients: selective mismatching for HLA B12, an antigen with variable expression on platelets. *Blood*. 1989;74(3):1172-1176.

22. Schipper RF, Schreuder GM, D'Amaro J, Oudshoorn M. HLA gene and haplotype frequencies in Dutch blood donors. *Tissue Antigens*. 1996;48(5):562-574.

23. Szatkowski NS, Aster RH. HLA antigens of platelets. IV. Influence of "private" HLA–B locus specificities on the expression of Bw4 and Bw6 on human platelets. *Tissue Antigens*. 1980;15(4):361-368.

24. Liebert M, Aster RH. Expression of HLA-B12 on platelets, on lymphocytes and in serum: a quantitative study. *Tissue Antigens*. 1977;9(4):199-208.

25. Aster RH, Szatkowski N, Liebert M, Duquesnoy RJ. Expression of HLA-B12, HLA-B8, w4, and w5 on platetelets. *Transplant Proc*. 1977;9(4):1695-1696.

26. Szatkowski N, Duquesnoy RJ, Aster RH. Further studies of the expression of BW4, BW6, and other antigens of the HLA-B locus on human platelets. Transpl Proc; 1978:9739-9740

27. Lim J, Kim Y, Han K, et al. Flow cytometric monocyte phagocytic assay for predicting platelet transfusion outcome. *Transfusion*. 2002;42(3):309-316.

28. van Hensbergen Y, Mulder A, Cornelissen JJ, Brand A. Validation of human monoclonal HLA Class I antibodies to evaluate the kinetics of donor chimerism in different cell subsets after double-cord-blood transplantation in the NOD/SCID model. *Transfusion*. 2013;53(1):104-114.

29. Mulder A, Kardol MJ, Arn JS, et al. Human monoclonal HLA antibodies reveal interspecies crossreactive swine MHC class I epitopes relevant for xenotransplantation. *Mol Immunol*. 2010;47(4):809-815.

30. Dellgren C, Ekwelum VAC, Ormhoj M, et al. Low constitutive cell surface expression of HLA-B is caused by a posttranslational mechanism involving Glu180 and Arg. *J Immunol*. 197(12):4807-4816.

31. René C, Lozano C, Villalba M, Eliaou J-F. 5′ and 3′ untranslated regions contribute to the differential expression of specific HLA-A alleles. *Eur J Immunol*. 2015;45(12):3454-3463.

32. Isa A, Nehlin JO, Sabir HJ, et al. Impaired cell surface expression of HLA-B antigens on mesenchymal stem cells and muscle cell progenitors. *PLoS One*. 2010;5(5):e10900.

33. Kelly AM, Garner SF, Herbert N, et al. The effect of variation in donor platelet function on transfusion outcome: a semi-randomised, double blind, controlled trial (PROmPT). *Blood*. 2014;124(21):595.