IMMUNOGENETICS

Original article

HLA class I expression on human platelets is highly variable and correlates with distinct allele group frequencies

Rocco Cantisani[1], Valeria Del Re[1], Francesca Toraldo[1], Silvia Cantara[2], Simone Pozzessere[1], Giuseppe Marotta[1], Adriano Spreafico[1]

[1]*Department of Cellular Therapies, Hematology and Laboratory Medicine, University Hospital of Siena, Siena, Italy;*
[2]*Department of Medical, Surgical and Neurological Sciences, University of Siena, Siena, Italy*

Background - Human leukocyte antigen (HLA) class I molecules are expressed on platelets and can represent a source of alloimmunization in recipients of platelet transfusions. HLA mismatch between donors and recipients may be associated with the induction of anti-HLA antibodies, which can culminate in refractoriness to platelet transfusions. In the present study we analyzed HLA allele group frequencies and HLA expression levels on human platelets from blood donors.

Materials and methods - Platelet-rich plasma was collected from 139 donors to monitor platelet HLA class I expression by flow cytometry. DNA from donors with high and low platelet HLA expression was used in the genotype studies. Frequencies of large and normal-sized platelet subpopulations were determined and HLA class I expression was studied. Mean platelet volume (MPV) and platelet large-cell ratio (P-LCR) were analyzed in both groups of donors.

Results - The analysis showed variable platelet HLA class I expression with significant differences among donors. HLA class I allele group frequencies in donors with high and low platelet HLA expression showed distinctive genotypic features strictly related to expression level. The main allele groups found in samples with high platelet HLA class I expression were HLA-A*02, -A*68, -B*15, -B*49, and -C*03. Platelet HLA class I expression did not change over time or during freezing-thawing cycles. The analysis of platelet subpopulations showed a statistically significant higher expression of HLA class I molecules on large platelets than on normal-sized platelets. Moreover, donors with high HLA class I expression showed a higher frequency of large platelets ($p<0.0001$). The analysis of P-LCR in both groups of donors showed a statistically significant difference ($p<0.05$) within high HLA-expressing donors.

Discussion - Our data suggest an allele-dependent expression of HLA class I molecules on human platelets with distinct HLA allele group frequencies and different platelet subpopulation frequencies among blood donors.

Keywords: *platelets, blood donors, HLA, alloimmunization, platelet transfusion.*

INTRODUCTION

Platelets are small, anucleated cells with a key role in hemostasis and the immune response to pathogens. Upon activation, platelets can release granule contents, inducing platelet aggregation and endothelial activation[1]. Platelets express Toll-like receptors

Arrived: 9 June 2023
Revision accepted: 21 September 2023
Correspondence: Rocco Cantisani
e-mail: rocco.cantisani@ao-siena.toscana.it

which are involved in the innate immune response, they can bind bacteria or internalize viruses directly and can release platelet microbicidal proteins. In their resting state, platelets have minimal ability to interact and stimulate a patient's immune system during transfusion, whereas upon activation they increase the expression of co-stimulatory molecules (CD80, CD86) and adhesion molecules which are involved in cellular interactions and in the stimulation of the immune response. The binding of Toll-like receptor 4 expressed by platelets induces the release of tumor necrosis factor-α and soluble CD40L. Platelets also synthesize and release interleukin (IL)-1β, IL-6, and IL-8 in both micro-vesicular and soluble forms. In addition, HMGB1, a DNA-binding protein that functions as a damage-associated molecular pattern, can be released by activated platelets in a soluble form or expressed at the cell surface, linking inflammation with hemostasis. However, since the effects of platelet activation induced by storage and platelet handling have been only partially defined, other studies are needed to obtain a better estimate of the impact of platelets on the recipient's immunoregulation after transfusion[2,3].

There are distinct platelet subsets with different functions, sizes and procoagulant capabilities. The functional heterogeneity allows platelets to assume distinct roles during the process of thrombosis. Compared to small platelets, large platelets aggregate faster and respond more strongly to agonists[4-7]. Platelet size can be determined by light, immunofluorescence and electronic microscopy or through analysis of forward scatter (FSC) in flow cytometry. In contrast, an automatic hematology analyzer assesses platelet size through the mean platelet volume (MPV) and the platelet-large cell ratio (P-LCR).

Platelet transfusions are used to prevent complications or treat critically ill patients. Circulating platelets express HLA class I molecules[8] and can therefore represent a source of alloimmunization[9,10]. In more detail, donor/recipient HLA mismatches can stimulate the immune system to produce specific anti-HLA antibodies, inducing rapid clearance of transfused platelets[11,12]. This scenario can culminate in refractoriness to platelet transfusions, defined as an inadequate platelet count increment after multiple platelet transfusions[13].

The presence of alloantibodies against HLA class I antigens determines platelet opsonization and, as a consequence, a fast decrease of transfused platelets via antibody- or complement-dependent cytotoxicity and/or antibody-dependent cellular phagocytosis[14-16]. The management of patients with platelet refractoriness is complex and the most important strategies include platelet cross-matching and the selection of donors with identical HLA[17,18]. Novel approaches include the selection of donor platelets with naturally low expression of HLA antigens in order to minimize platelet opsonization[19]. However, the level of expression of HLA class I molecules is not static and can be influenced by multiple factors (DNA methylation or post-transcriptional regulation)[20]. Interestingly, conclusive data on whether HLA class I expression on human platelets correlates with specific HLA allele group frequencies are lacking.

MATERIALS AND METHODS

Samples

Healthy male or female donors aged 18 years or older were randomly selected from the daily blood donation list. Whole venous peripheral blood was obtained from a total of 139 donors. Informed consent to participation in the study had been signed by each donor at the time of the blood donation. The platelet donors in our study were representative of a large cohort of HLA fully typed bone marrow donors (more than 1,500) enrolled in our Transfusion Unit with the same eligibility criteria. A comparison of HLA frequencies with those of the Italian population reported in the Allele Frequency Net Database (*http://allelefrequencies.net*), chosen as the reference population, did not show any significant differences. The study was approved by our local Ethical Committee (Ethical Committee of Region Toscana, Area Vasta Sud Est, AOUS. Protocol ID: 10167).

Blood samples were analyzed with a hematology analyzer (Sysmex XN-550, Myco Instrumentation Inc., Bonney Lake, WA, USA) which measures platelet count, MPV and P-LCR. HLA class I expression on platelets was analyzed by flow cytometry (BD Facs Canto II, BD Bioscience, Franklin Lakes, NJ, USA) using platelet-rich plasma (PRP) from each donor. To determine HLA allele group frequencies, DNA from blood samples was extracted, amplified by polymerase chain reaction and analyzed by Luminex.

HLA class I expression analysis

Blood samples were centrifuged at 100 × *g* for 10 minutes and then PRP was collected and stored at –20°C or analyzed directly. For direct analysis, 2 mL of phosphate-buffered saline (PBS)/ethylenediaminotetraacetic acid (EDTA) 5mM were added to 200 µL of PRP and centrifuged at 700 × *g* for 7 minutes. After centrifugation the supernatant was discarded and the pellet was gently resuspended in 2 mL of PBS/EDTA 5mM. Then 100 µL were incubated with 20 µL of anti-HLA ABC-PE (BD Pharmingen clone G46-2.6) or IgG-PE Isotype control (BD Pharmingen clone MOPC-21) together with 20 µL of anti CD42b-APC (BD Pharmingen clone HIP1). After 20 minutes of incubation at room temperature, samples were washed twice with 2 mL of PBS/EDTA 5mM with centrifugation at 700 × *g* for 7 minutes. Finally, the pellet was gently resuspended with 1 mL of PBS/EDTA 5mM and analyzed by flow cytometry. For each sample 50,000 events were acquired.

Platelet HLA expression was determined by analyzing the mean fluorescence intensity (MFI) of HLA ABC-PE gated on CD42b-positive cells. An experimental curve from flow cytometry data of platelet HLA expression was built and fitted using "CurveExpert" software. The calculated slope changes were used to arbitrarily select two donor groups (with high or low HLA expression) for the following genotype analyses. To analyze HLA expression on platelet subpopulations, platelet populations with higher FSC (large platelets) and lower FSC (normal-sized platelets) were selected and HLA MFI values were determined.

HLA typing

DNA from donor blood samples was extracted using an EZ1 DNA Blood Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and quantified by a NanoDrop Lite Spectrophotometer (ThermoScientific, Waltham, MA, USA) for the genotype analyses. HLA class I low resolution typing (HLA-A, HLA-B, HLA-C loci) was performed by a sequence-specific oligonucleotide (SSO) probe-based hybridization technique, using an Immucor (Norcross, GA, USA) SSO-Kit (Lifecodes HLA SSO typing kits) in accordance with the manufacturer's instructions. After polymerase chain reaction amplification, samples were analyzed by Luminex (LS200). Data on allele frequencies of class I HLA genes among the reference population were obtained from the Allele Frequency Net Database. The reference population used in this study was Italian population 5, Italy country, Caucasian ethnic origin.

Statistical analysis

GraphPad Prism 9 software (GraphPad Software, Boston, MA, USA) was used to analyze the experimental data. Statistical differences were tested using a Student's t-test for flow cytometry data. The HLA-A, -B and -C allele group frequencies were obtained by the direct counting method. A χ^2 test was used to compare the allele group frequencies in the study population with those in the reference population. p values were considered statistically significant below 0.05 (*p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001).

RESULTS

HLA class I expression on human platelets differs between donors

To study the variability of HLA class I surface expression on human platelets we analyzed PRP from 139 randomly selected blood donors by flow cytometry. Platelets were identified first with a morphological gate and then for the expression of the CD42b marker. HLA-ABC MFI values of the platelet populations were collected and analyzed (**Figure 1A**), which showed a significant variability in HLA class I molecule expression among donors, with MFI values ranging between 6,000 and 20,000 (**Figure 1B**). Given the wide variability of HLA expression, we arbitrarily defined two groups of donors based on curve slope changes: a group with higher HLA expression (MFI >15,000) and a group with lower HLA expression (MFI <10,000) (**Figure 1C**). As reported, a marked difference (p<0.0001) was observed between the two groups, confirming the wide variability of HLA class I expression on platelets among donors. We then investigated whether different blood donations could affect HLA class I expression. To address this question, HLA class I expression was monitored in four different donors during three independent platelet apheresis and no major changes in HLA class I expression were observed (**Figure 1D**). Finally, we compared the analysis of frozen PRP *vs* thawed PRP in order to explore different experimental conditions. As shown in **Figure 1E**, the analysis of frozen *vs* fresh stained PRP did not reveal any differences between the two storage conditions. These data suggest that HLA class I molecule expression on human platelets is highly variable between donors and can be monitored over time.

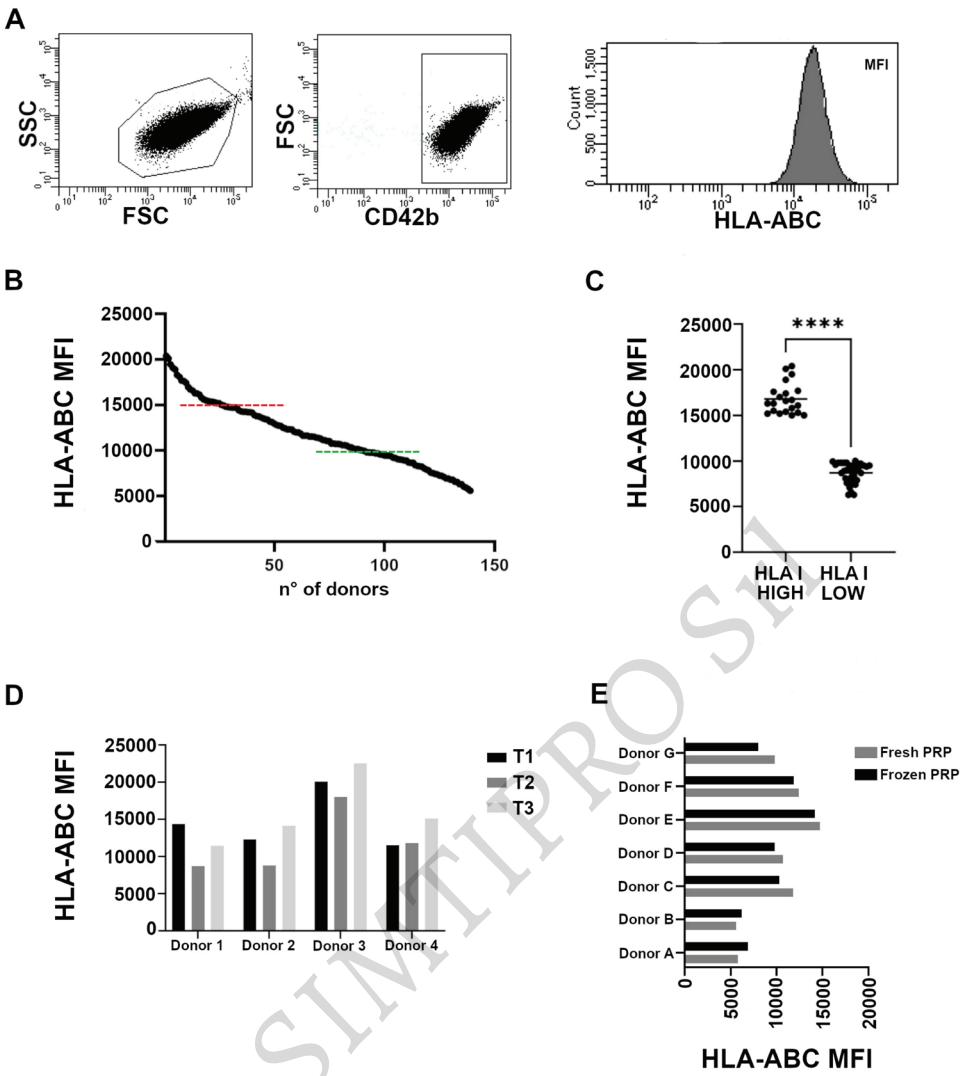

Figure 1 - HLA class I expression on human platelets differs between donors
(**A**) Gating strategy for the identification of platelets by flow cytometry. Platelets from platelet-rich plasma (PRP) were detected first through a morphological gate (side scatter/forward scatter) and then for the expression of the CD42b marker. Mean fluorescence intensity (MFI) of HLA-ABC loci was analyzed. (**B**) HLA-ABC MFI of platelets from blood donors (No.=139) was plotted from highest to lowest values. Curve slop changes are marked with red and green lines. (**C**) Platelet donors with HLA-ABC MFI >15,000 (high expression donors, No.=27) and platelet donors with HLA-ABC MFI <10,000 (low expression donors, No.=24) were compared. A statistically significant difference was observed (p<0.0001). (**D**) Platelet HLA-ABC MFI values were analyzed in four different donors at three independent platelet aphereses (T1, T2, T3). The coefficient of variation (CV) was calculated for each donor at each time point showing a CV <0.5. (**E**) Platelet HLA-ABC MFI values of fresh PRP vs frozen/thawed PRP were analyzed in seven different donors. FSC: forward scatter; SSC: side scatter.

Donors with low and high HLA class I platelet expression display distinct allele group frequencies

In order to understand the distribution of HLA class I alleles in platelet donors, we studied HLA genotype frequencies comparing donor platelets with high and low HLA class I expression. DNA from the corresponding donors was extracted from whole blood, amplified for HLA-A, -B, and -C loci and analyzed by Luminex. Allele group frequencies for the HLA-A, -B and -C loci are shown in **Table I**. We identified 12 HLA-A, 21 HLA-B and 13 HLA-C allele groups. As expected, the highest polymorphism was observed within the HLA-B region. The analysis of donors with high HLA class I platelet expression showed five HLA-A allele groups (-A*02, -A*24, -A*32, -A*33, -A*68), four HLA-B allele groups (-B*15, -B*18, -B*45, -B*49) and four HLA-C allele groups (-C*02, -C*03, C*04, -C*16)

with statistically significant differences of frequencies compared to those of the reference population. These statistically significant differences were not found in the group of donors with low HLA class I platelet expression, indicating a predominant distribution of the indicated allele groups strictly related to high HLA class I expression. Of note HLA-A*68, -B*45 and -B*49, were never found in the low HLA expression group.

The HLA-B*15 allele family displays a multiplicity of antigenic specificities. In our study the most common HLA-B*15 specificity observed in high HLA-expressing donors was -B*15:01. We also observed that the frequency of HLA-A*33, -B*18 and -C*04 was significant in the two groups of donors (high and low HLA expression) indicating a possible connection with the level of expression of other HLA specificities. Lastly, even if HLA-B*27, -B*53, -B*58, -C*01, and -C*14 were never found in low HLA class I expression donors, we observed similar frequencies compared to those in the reference population. Conversely, the analysis of donors with low HLA class I platelet expression showed the presence of five different HLA-A allele groups (-A*01, -A*03, -A*11, -A*30, -A*33), six HLA-B allele groups (-B*08, -B*18, -B*35, -B*37, -B*38, -B*47) and three HLA-C allele groups (-C*04, -C*06, -C*12) with statistically significant differences of frequencies compared to those in the reference population. These statistical differences were not found in donors with high HLA class I platelet expression indicating a predominant distribution of the indicated allele groups strictly connected with low HLA class I expression. Of note, HLA-B*08, -B*37, -B*38 and -B*47 were never found in high HLA expression donors. Lastly, HLA-B*41 and -B*57, although never found in high HLA class I-expressing donors we did not observe major changes in frequency compared to the reference population. Thus, our study revealed an association in human platelets between *in vivo* HLA class I expression and HLA frequencies with distinct genotypic features.

Table I - *Allele group frequencies of HLA-A, HLA-B and HLA-C loci in donors with high HLA platelet expression (HLA high, No.=27) and low HLA platelet expression (HLA low, No.=24) compared to the reference population (ITA population)*

HLA-A					
Allele group	**ITA population frequency**	**HLA high frequency**	**HLA high p-value**	**HLA low frequency**	**HLA low p-value**
A*01	0.121	0.037	0.886999	0.187	**
A*02	0.25	0.277	****	0.145	0.999972
A*03	0.114	0.037	0.550791	0.229	***
A*11	0.06	0.055	0.263463	0.1	**
A*24	0.122	0.129	*	0.02	0.237656
A*26	0.05	0.018	0.757343	0.041	0.453695
A*29	0.036	0.037	0.179487	0.041	0.125663
A*30	0.05	0.018	0.757343	0.125	***
A*31	0.025	0.037	0.10243.	0.02	0.6009
A*32	0.051	0.092	**	0.06	0.453695
A*33	0.022	0.055	***	0.041	*
A*68	0.039	0.111	****	-	-
HLA-B					
Allele group	**ITA population frequency**	**HLA high frequency**	**HLA high p-value**	**HLA low frequency**	**HLA low p-value**
B*07	0.057	0.037	0.701915	0.02	0.746008
B*08	0.059	-	-	0.1	*
B*13	0.034	0.037	0.250582	0.041	0.182371
B*14	0.036	0.037	0.288302	0.041	0.213285
B*15	0.052	0.222	****	0.02	0.819667
B*18	0.097	0.111	*	0.125	*
B*27	0.019	0.018	0.492277	-	-
B*35	0.159	0.148	0.085293	0.27	****
B*37	0.014	-	-	0.06	****
B*38	0.035	-	-	0.06	*
B*40	0.027	0.037	0.13132	0.02	0.657521
B*41	0.01	-	-	0.02	0.248692
B*44	0.092	0.074	0.312791	0.06	0.883261
B*45	0.005	0.018	*	-	-
B*47	0.004	-	-	0.041	****
B*49	0.036	0.055	****	-	-
B*51	0.104	0.092	0.14015	0.041	0.785508
B*53	0.009	0.018	0.957997	-	-
B*55	0.026	0.018	0.718579	0.02	0.629624
B*57	0.049	-	-	0.02	0.867851
B*58	0.035	0.03	0.943995	-	-

HLA-C					
Allele group	**ITA population frequency**	**HLA high frequency**	**HLA high p-value**	**HLA low frequency**	**HLA low p-value**
C*01	0.045	0.037	0.2366	-	-
C*02	0.015	0.055	*	0.02	0.53501
C*03	0.035	0.185	****	0.02	0.970266
C*04	0.15	0.2	*	0.2	*
C*05	0.04	0.037	0.458982	0.06	0.105169
C*06	0.1	0.037	0.682778	0.16	*
C*07	0.3	0.2	0.156883	0.18	0.936137
C*08	0.03	0.037	0.296039	0.02	0.771436
C*12	0.14	0.018	0.141293	0.187	*
C*14	0.03	0.2	0.296039	-	-
C*15	0.03	0.018	0.85262	0.02	0.771436
C*16	0.035	0.07	*	0.041	0.374311
C*17	0.005	0.018	0.316672	0.02	0.268761

The symbol "-" is used to represent cases in which the corresponding HLA allele was not detected. *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001.

Subpopulations of large platelets express more HLA class I molecules

There is increasing evidence about the different biological roles of platelet subpopulations. However, their immunological functions are still unknown and not completely understood. In order to understand whether HLA class I molecules are differently expressed among platelet subpopulations, we compared platelets with higher FSC (large platelets) with platelets with lower FSC (normal-sized platelets) in high and low HLA class I-expressing donors by analyzing HLA-ABC MFI values (**Figure 2A**). As shown in **Figure 2B** we observed higher expression of HLA class I molecules on large platelets than on normal-sized platelets from donors with high HLA expression, with a statistically significant difference ($p<0.0001$). The difference in high HLA class I expression of large platelets compared to the other platelet population was still maintained in low-HLA expressing donors ($p<0.0001$). Our data are in line with published data about the higher HLA expression of large platelets.

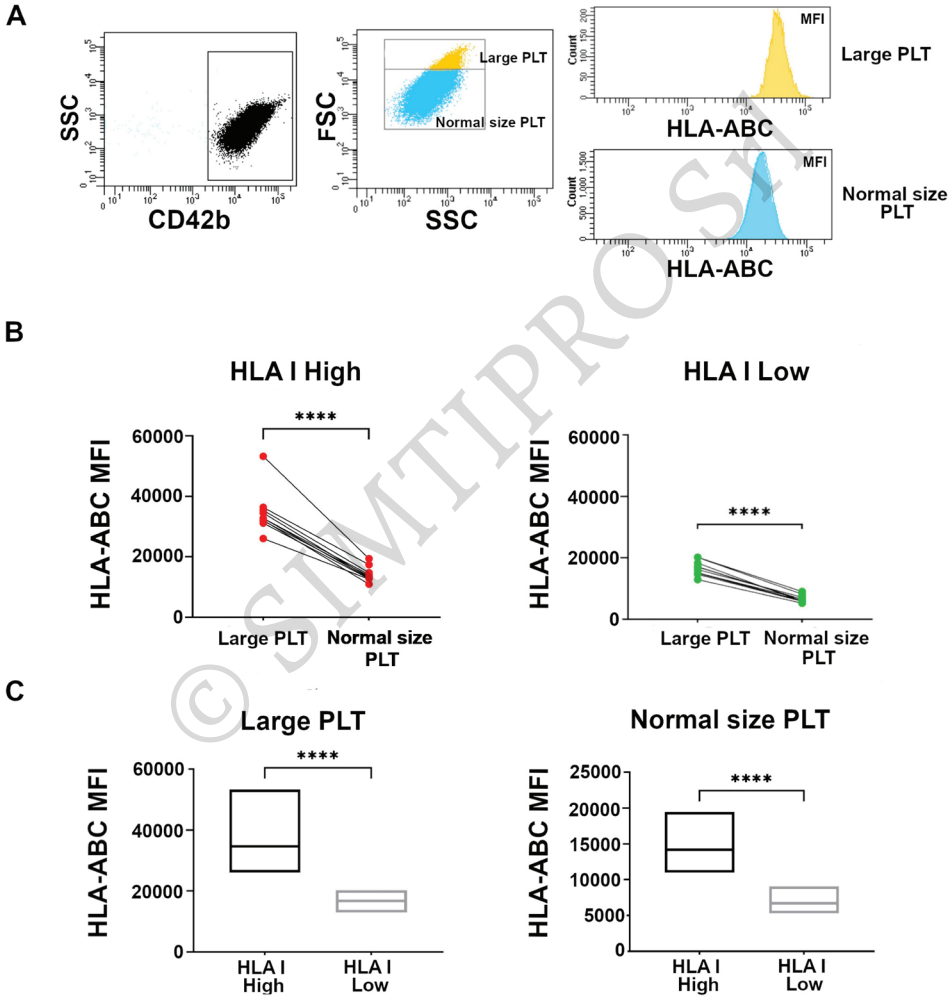

Figure 2 – Subpopulations of large platelets express more HLA class I molecules

(**A**) Gating strategy for the identification of platelet subpopulations by flow cytometry. Platelets from platelet-rich plasma were detected through expression of the CD42b marker. Platelet subpopulations with higher forward scatter (large platelets) or lower forward (normal-sized platelets) were identified. HLA-ABC mean fluorescent intensity (MFI) values of both platelet populations were analyzed. (**B**) Large or normal-sized platelets were analyzed for HLA class I expression in donors with high HLA class I expression (left panel, No.=10) and low HLA class I expression (right panel, No.=10). A statistically significative difference was observed ($p<0.0001$) comparing HLA-ABC MFI values in the two group of donors. (**C**) Box-whisker plots of mean values of HLA-ABC MFI comparing the populations of large platelets (left panel, No.=10) with the normal-sized platelets (right panel, No.=10) in high- and low-HLA expressing donors. A statistically significant difference was observed ($p<0.0001$). SSC: side scatter; FSC: forward scatter; PLT: platelets.

As expected, the comparison of large and normal-sized populations of platelets in both groups of donors (high vs low HLA expression) showed a statistically significant difference ($p<0.0001$) suggesting a close correlation with the level of expression (**Figure 2C**). Overall, these data highlight novel features of platelet subpopulations in blood donors with differential HLA class I expression between platelet subsets.

Donors with high HLA class I platelet expression show higher frequencies of large platelets

In order to explore platelet subpopulations among blood donors, frequencies of large and normal-size platelets were studied. We analyzed the percentage of $CD42b^+$ platelets comparing both platelet populations in high and low HLA class I-expressing donors. As illustrated in **Figure 3A** we found a statistically significant difference in the percentage of large platelets in high-HLA-expressing donors ($p<0.001$) compared to normal sized platelet frequency. Conversely, no differences were observed in the percentage of normal-sized platelet population of both groups of donors. Our data suggest that donors with high HLA expression have more circulating large platelets compared to low expression donors.

Given the differences in platelet subpopulation frequencies, we investigated whether any platelet parameter differed between the two groups. Using a hematology analyzer we studied blood samples from high- and low-HLA-expressing donors, focusing our attention on MPV and P-LCR (**Figure 3B**). Interestingly, although we did not observe differences in MPV values between donors (high vs low HLA expression), we did note a statistically significant higher P-LCR ($p<0.05$), an indicator of circulating larger platelets, in high HLA-expressing donors. Our study indicates a possible correlation between P-LCR and donor platelets with high HLA class I expression.

DISCUSSION

The aim of this study was to explore the expression profile of HLA class I molecules on human platelets from randomly selected blood donors. HLA alloimmunization is the most important cause of platelet refractoriness and can be a clinically significant problem[21-23]. The frequency of HLA alloimmunization varies between patients given multiple transfusions, but there is no apparent dose-response relationship between the number of platelet transfusions and immunization of patients[12]. The mechanisms that result in stimulating immunity against blood products are still incompletely understood. We observed a large difference in platelet HLA expression even though we did not discriminate the expression of A, B and C loci separately. Flow cytometry analysis

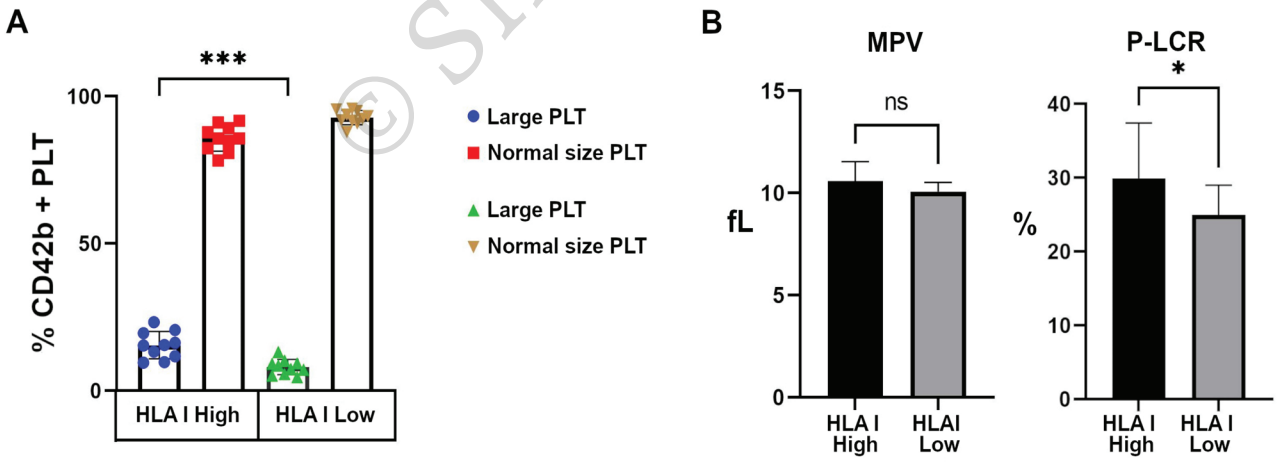

Figure 3 - Donors with high HLA class I expression show higher frequencies of large platelets
(**A**) Frequencies of CD42b+ large platelets (blue circles) and normal-sized platelets (red squares) in high HLA-expressing donors (left panel, No.=10) are illustrated. The right panel shows the frequencies of CD42b+ large platelets (green triangles) and normal-sized platelets (brown triangles) in low HLA-expressing donors (No.=10). Where reported, a statistically significant difference was observed ($p<0.001$). (**B**) The platelet parameters, mean platelet volume (MPV, left panel) and platelet-large cell ratio (P-LCR, right panel), were analyzed in high HLA-expressing donors (HLA class I high, No.=27) and low HLA-expressing donors (HLA class I low, No.=24). The mean value and standard deviation are reported. No statistical differences were observed in MPV (p=ns) while a statistically significant difference was observed in P-LCR ($p<0.05$). PLT: platelets.

quickly provides information on the level of expression of total HLA class I molecules[24]. In particular we identified donors with high HLA expression and donors with low HLA expression who were selected for genotype analysis. The analysis of platelet HLA expression in different donations from the same donor highlighted that the expression of HLA class I molecules on platelets remains almost unchanged over time. HLA typing could allow characterization and classification of platelet donors for their different HLA class I expression.

Although it has not yet been determined whether there is a connection between HLA expression profile and transfusion yield, the transfusion of platelet concentrates with distinct cellular characteristics could open novel scenarios in the field of transfusion medicine. Interestingly, molecular typing showed different allele group frequencies between donor platelets with high and low HLA class I expression. In particular our objective was to determine whether HLA specificities could correlate with the level of platelet expression. We found that HLA-A*02, -A*32, -A*24 and -A*68 were mainly distributed in donors with high HLA class I expression while HLA-A*01, -A*03, -A*11, and -A*30 were present among donors with low HLA class I expression. The genotypic analysis of HLA B loci showed that HLA-B*15, -B*45 and -B*49 were the predominant allele groups in high-HLA-expressing donors while, conversely, HLA-B*08, -B*35, -B*37, -B*38 and -B*47 were mainly present among low-HLA-expressing donors.

The majority of Italian studies reported to date refer to HLA-A, HLA-B or HLA-DR polymorphisms while there are only few data on the molecular diversity of HLA-C. The paucity of interest reserved to HLA-C studies may be due to a lower number of allelic variants of this locus and its lower immunogenic profile compared to the other HLA class I loci. We observed the presence of HLA-C*02, -C*03, and -C*16 allele groups mostly in donors with high HLA class I expression, while HLA-C*06 and -C*12 were the predominant allele groups in low HLA class I-expressing donors. Our study therefore revealed a possible connection between HLA class I molecule expression and allele distribution in human platelets.

Of note, HLA-A*33, -B*18 and -C*04 allele groups were found at significant frequencies in both groups of donors. Since we analyzed the global expression level of HLA class I loci, it is possible that the level of expression of other specificities could interfere or mask the real contribution of these allele groups within the two groups. For this reason we did not consider these HLA allele specificities distinctive of a specific group. It was recently reported that donors with low expression of HLA-B*08, -B*12 and -B*35 on human platelets showed decreased antibody-mediated internalization by macrophages[19]. The selection of donors with low levels of HLA class I molecules is therefore an attractive strategy for the management of platelet refractory patients. However, even if it has not yet been demonstrated in vivo that high levels of HLA molecules could have a major impact on alloimmunization, we can assume that the amount of HLA antigens on platelets is closely related to the induction of anti-HLA antibody production. On this background, new randomized clinical trials of refractory patients should be performed to evaluate the transfusion effects of platelets with high or low HLA expression.

To further investigate the relevance of platelets in the context of transfusion medicine we analyzed platelet subpopulations by flow cytometry. In our experimental conditions we were able to discriminate two platelet populations based on FSC differences. In particular we analyzed a fraction with higher FSC (large platelets) and another fraction with lower FSC (normal-sized platelets). We analyzed both platelet populations among donors with high and low HLA class I expression. Recent studies indicate that large platelets are functionally different since they were identified as a subpopulation with higher pro-hemostatic capacity, larger amounts of proteins and more HLA class I molecules[7,8,25,26]. However, although large platelets differ for their physiological and immunogical properties, it is not well established whether this subpopulation is involved in specific diseases or pathological disorders. The pro-thrombotic features of large platelets seem to play a role in cardiovascular disease and thrombotic complications primarily in critically ill patients. In addition, the higher expression of HLA class I molecules on large platelets has not been fully investigated in the context of immune system modulation together with the different antigen-presenting properties of specific HLA allele groups. Future studies are needed to address the role of differently sized platelet subpopulations on host immunity and pathophysiological states. In line with published data we observed higher expression of HLA class

I molecules on large platelets compared to normally sized platelets. Interestingly we also observed that frequencies of large platelets were statistically higher in donors with high HLA expression than in those with low HLA expression donors, which correlated with P-LCR values.

In summary our study revealed a close correlation between HLA class I expression levels and allele group frequencies in platelet donors. Future studies are needed to evaluate how HLA expression level can influence patients' immune responses during platelet transfusions and whether the use of low-HLA expression platelet concentrates could prevent platelet refractoriness.

The possibility of analyzing different platelet subpopulations could open up new perspectives for the selection of donors with specific cellular features. Flow cytometry may be an attractive option to monitor additional platelet parameters. In this direction it would be interesting to explore two aspects: first, the relationship between transfusion yield and different HLA expression levels together with the immunogenicity of HLA specificites; and second, what is the pro-coagulant capacity of different platelet subpopulations in a transfusion context. In the perspective of implementation in blood bank laboratories we think that these insights could be useful for clinicians involved in the management of critically ill patients.

CONCLUSIONS

In this study we analyzed whether there is a possible link between HLA expression and allele group frequency in platelet donors and explored platelet subpopulations in the donors. Since large platelets may be classified as a pro-hemostatic subpopulation, the selection of donors with naturally high HLA class I expression could enable the production of platelet concentrates enriched for the fraction of large platelets, which should be helpful for the treatment of specific patients, e.g., trauma patients. In contrast, methods to enrich the fraction depleted of large platelets from donors with naturally low HLA class I expression could be implemented in order to produce platelet concentrates mainly indicated for the management of refractory patients. Furthermore donor-specific anti-HLA antibodies are a major cause of engraftment failure in patients undergoing haploidentical stem cell transplantation. Effective treatments are needed for these patients who often have no other donor options and/or need to proceed urgently to transplantation[27]. The transfusion of platelet concentrates with a specific level of HLA molecules could be used preventively to adsorb anti-HLA antibodies in patients before HLA-mismatched transplantation.

In conclusion, the possibility of selecting donors with specific characteristics together with the use of novel techniques for the enrichment of a specific platelet population could open new scenarios in transfusion medicine and might be relevant for the outcomes of transfused patients.

ACKNOWLEDGMENTS

We would like to thank the medical staff of the Transfusion Medicine Department of the University Hospital of Siena for collecting informed consent from the blood donors, Monica Tozzi and Alessandro Bucalossi for critical discussion of data, and Enza Frigidi for the helpful support with flow cytometry.

ETHICAL CONSIDERATIONS

This study was approved by the Institutional Ethics Committee of Region Toscana, Area Vast-a Sud Est, AOUS Protocol ID: 10167. The research was conducted ethically, with all study procedures being performed in accordance with the requirements of the World Medical Association's Declaration of Helsinki. Written informed consent to participation in the study and data publication was obtained from each blood donor.

AUTHORSHIP CONTRIBUTIONS

RC performed the molecular and flow cytometry tests, analyzed the results and wrote the paper. VDR performed flow cytometry tests and reviewed the paper. FT, SC, SP, GM, and AS critically reviewed the manuscript. AS and GM contributed to designing the study. AS evaluated the data.

The Authors declare no conflicts of interest.

REFERENCES

1. Maui M, Rebetz J, Kapur R, Semple JW. The immune nature of platelets revisited. Trans Med Rev 2020; 34: 209-220. doi: 10.1016/j.tmrv.2020.09.005.
2. Gros A, Oliver V, Ho-Tin-Noe B. Platelets in inflammation: regulation of leukocyte activities and vascular repair. Front Immunol 2014; 5: 678. doi: 10.3389/fimmu.2014.00678.

3. Gaertner F, Massberg S. Patrolling the vascular border: platelets in immunity to infection and cancer. Nat Rev Immune 2019; 19: 747-760. doi: 10.1038/s41577-019-0202-z.
4. Agbani EO, Poole AW. Procoagulant platelets: generation, function and therapeutic targeting in thrombosis. Blood 2017; 130: 2171-2179. doi: 10.1182/blood-2017-05-787259.
5. Tynngard N, Alshamari A, Sandgren Per, Kenny D, Vasilache AM, Abedi MR, et al. High fragmentation in platelet concentrates impacts the activation, procoagulant, and aggregatory capacity of platelets. Platelets 2023; 34: 2159018. doi: 10.1080/09537104.2022.2159018.
6. Storrie B. A tip of the cap to procoagulat platelets. Blood 2016, 128: 1668-1669. doi: 10.1182/blood-2016-08-730622.
7. Handtke S, Thiele T. Large and small platelets-(when) do they differ? J Thromb Haemost 2020; 18: 1256-1267. doi: 10.1111/jth.14788.
8. Pereira J, Cretney C, Aster RH. Variation of class I HLA antigen expression among platelet density cohorts: a possible index of platelet age? Blood 1988; 2: 516-519. PMID: 2962651
9. Chapman LM, Aggrey AA, Field DJ, Srivastava K, Ture S, Yui K, et al. Platelets present antigen in the context of MHC class I. J Immunol 2012; 189: 916-923. doi: 10.4049/jimmunol.1200580.
10. Garraud O, Cognasse F, Moncharmont P. Immunological features in the process of blood platelet-induced alloimmunisation, with a focus on platelet component transfusion. Disease 2019; 7: 7. doi: 10.3390/diseases7010007.
11. Grozovsky R, Hoffmeister KM, Faset H. Novel clearance mechanisms of platelets. Curt Opin Hematol 2010; 17: 585-589. doi: 10.1097/MOH.0b013e32833e7561.
12. Pavenski K, Freedman J, Semple JW. HLA alloimmunization against platelet transfusions: pathophysiology, significance, prevention and management. Tissue Antigens 2012; 79: 237-245. doi: 10.1111/j.1399-0039.2012.01852.x.
13. Comont T, Tavitian S, Bardiaux L, Fort M, Debiol B, Morère D, et al. Platelet transfusion refractoriness in patients with acute myeloid leukemia treated by intensive chemotherapy. Leuk Res 2017; 61: 62-67. doi: 10.1016/j.leukres.2017.08.015.
14. Badlou BA, Ya PW, Smid WM, Akkerman JWN. Platelet binding and phagocytosis by macrophages. Transfusion 2006; 46: 1432-1443. doi: 10.1111/j.1537-2995.2006.00913.x.
15. Meinke S, Karlstrom C, Hoglund P. Complement as an immune barrier in platelet transfusion refractoriness. Trans Med Rev 2019; 33: 231-235. doi: 10.1016/j.tmrv.2019.09.003.
16. Van Osch TLJ, Steuten J, Nouta J, Koeleman C, Bentlage AEH, Hedt S, et al. Phagocytosis of platelets opsonized with differently glycosylated anti-HLA hIgG1 by monocyte-derived macrophages. Platelets 2023; 34: 1-9. doi: 10.1080/09537104.2022.2129604.
17. 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: 1959-1965. doi: 10.1111/j.1537-2995.2008.01799.x.
18. Stanworth SJ, Navarrete C, Estcourt L, Marsh J. Platelet refractoriness-practical approaches and ongoing dilemmas in patient management. Br J Haematol 2015; 171: 297-305. doi: 10.1111/bjh.13597.
19. Saris A, Tomson B, Brand A, Mulder A, Claas FH, Lorinser J, et al. Platelets from donors with consistently low HLA-B8, -B12, or -B35 expression do not undergo antibody-mediated internalization. Blood 2018; 131: 144-152. doi: 10.1182/blood-2017-07-799270.
20. Carey SB, Poulton KV, Poles A. Factors affecting HLA expression: a review. Intern Jour Immunogen 2019; 46: 307-320. doi: 10.1111/iji.12443.
21. Brown CJ, Navarrete CV. Clinical relevance of the HLA system in blood transfusion Vox Sang 2011; 101: 93-105. doi: 10.1111/j.1423-0410.2011.01474.x.
22. Saris A, Pavenski K. Human Leucocyte Antigen Alloimmunization and Alloimmune Platelet Refractoriness. Transf Med Rev 2020; 34: 250-257. doi: 10.1016/j.tmrv.2020.09.010.
23. Couvidou A, Rojas-Jiménez G, Dupuis A and Maitre B. Anti-HLA class I alloantibodies in platelet transfusion refractoriness: from mechanisms and determinants to therapeutic prospects. Front Immun ol 2023; 14: 1125367. doi: 10.3389/fimmu.2023.1125367.
24. Raghavan M, Yarzabek B, Zaitouna AJ, Krishnakumar S, Ramt DS. Strategies for the measurements of expression levels and half-lives of HLA class I allotypes. Hum Immunol 2020; 80: 221-227. doi: 10.1016/j.humimm.2019.02.001.
25. Veninga A, Handtke S, Aurich K, Tullemans BME, Brouns SLN, Schwarz SL, et al. GPVI expression is linked to platelet size, age, and reactivity. Blood Adv 2022; 6: 4162-4173. doi: 10.1182/bloodadvances.2021006904.
26. Clancy L, Beaulieu LM, Tanriverdi K, Freedman JE. The role of RNA uptake in platelet heterogeneity. Thromb Haemost 2017; 117: 948-961. doi: 10.1160/TH16-11-0873.
27. Yamashita T, Ikegame K, Kojima H, Tanaka H, Kaida K, Inoue T, et al. Effective desensitization of donor-specific HLA antibodies using platelet transfusion bearing targeted HLA in a case of HLA-mismatched allogeneic stem cell transplantation. Bone Marrow Transp 2017; 52: 794–796. doi: https://doi.org/10.1038/bmt.2017.10