Received: 22 November 2020 | Revised: 29 April 2021 | Accepted: 18 May 2021

DOI: 10.1002/jcla.23864

RESEARCH ARTICLE

WILEY

Transfusion-induced platelet antibodies and regulatory T cells in multiply transfused patients

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Funding information
This study was supported by a grant from Zhejiang Province Public Welfare Technology Application Research Project (grant number LGD19H080002) and Open Foundation of Key Laboratory of Blood Safety Research of Zhejiang Province (grant number 2020KF002)

Abstract

Background: Platelet transfusion refractoriness (PTR) remains a difficult problem in patients requiring long-term platelet supportive care. However, there are little data on the frequency of platelet antibodies in multiply transfused Chinese patients. Moreover, the relationship between peripheral regulatory T cells (Tregs) and PTR remains unclear.

Methods: We retrospectively studied the frequency of alloimmunization against platelet antigens in patients receiving multiple transfusions between 2013 and 2017. Monoclonal antibody solid-phase platelet antibody test (MASPAT) kits were used to screen for platelet antibodies before each platelet transfusion. Peripheral Tregs and $CD4^+CD25^+CD127^-$ T cells were detected by flow cytometry, while transforming growth factor-beta (TGF-β) and interleukin (IL)-17 cytokines were detected by enzyme-linked immunosorbent assay.

Results: A total of 399 patients who met the inclusion criteria were enrolled for the analysis of platelet antibodies and refractoriness. Among these patients, 10 (2.5%) were positive for platelet antibodies before transfusion and 47 (11.8%) became antibody-positive during the study period. The number of alloimmunized patients was significantly higher in patients with hematological disease as compared with other disease groups ($p < 0.05$). Refractoriness and alloimmunization occurred in 77 (19.3%) and 22 (28.6%) patients, respectively. There were no significant differences in $CD4^+$, $CD8^+$, and $CD4^+CD25^+CD127^-$ T cell numbers and plasma levels of TGF-β1 and IL-17 between patients with PTR and the control group.

Conclusions: Refractoriness was common in patients undergoing multiple platelet transfusions (19.3%), with alloimmunization observed in 28.6% of patients. However, Tregs in peripheral blood may not play a key role in PTR.

KEYWORDS
alloimmunization, platelet antibodies, platelet refractoriness, platelet transfusions, regulatory T cells

1 | INTRODUCTION

Platelet (PLT) transfusion is an essential therapy to maintain hemostasis in patients with thrombocytopenia. However, one sequela of PLT transfusion therapy is alloimmunization to donor PLT antigens, predominantly human leukocyte antigen (HLA) class I and, in some cases, human PLT antigens (HPA), and CD36. Platelet antibodies, which are directed against the HLA, HPA, and CD36 present on the

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platelet surface, can create a refractory state, whereby transfused PLTs are rapidly cleared in the recipient due to opsonization by antibody binding.[1,2]

However, the mechanisms underlying cellular responses that result in alloantibody production after allogeneic PLT transfusion as well as the effects of alloantibodies on immune-mediated PLT refractoriness remain poorly understood.[3,4]

Alloimmunization to transfused PLTs requires priming and activation of $CD4^+$ T cells specific for peptides derived from platelet antigens. These T cells in turn stimulate B cells to differentiate into plasma cells that produce immunoglobulin G (IgG) antibodies that bind PLT surfaces, leading to platelet transfusion refractoriness (PTR).[5] Regulatory T cells (Tregs) contribute to the maintenance of peripheral immune tolerance; defects in these are thought to play a role in the pathogenesis of autoimmune diseases.[6] The molecular basis of the inhibition of $CD4^+$ T cell activation and proliferation may be mediated by the secretion of the inhibitory cytokines interleukin (IL)-10 and transforming growth factor-beta (TGF-β). Tregs are essential regulators of self-tolerance and directly suppress acquired immune responses in the periphery.[7] Recent studies have increased the understanding of the production of PLT alloantibodies and its associations with Tregs;[6,8] however, it is not fully elucidated to what extend the proportion of Tregs and related cytokines in peripheral blood is associated with PTR.

Previous clinical studies have described the relationships between the number of PLT transfusions, PLT alloimmunization, and refractoriness in multiply platelet-transfused patients. The Trial to Reduce Alloimmunization to Platelets (TRAP) involved administration of four types of PLTs. Approximately 17% of patients with acute myeloid leukemia receiving leukoreduced, single-donor, apheresis PLTs during induction therapy became alloimmunized; 8% developed PTR.[9] However, only 5% of patients became alloimmunized in the Prophylactic Platelet Dose on Transfusion Outcomes (PLADO) trial.[10,11]

A total of 52 HLA-A, 96 HLA-B, and 61 HLA-DRB1 alleles were found in the Zhejiang Chinese Han population.[12] However, to our knowledge, baseline data are limited regarding the incidence of PLT alloimmunization and refractoriness in patients in China.[13]

This report assessed the development of PLT antibodies in previously untreated patients receiving PLT transfusions and examined the associations of T lymphocyte, B lymphocyte, TGF-β, and IL-17 levels in peripheral blood with PTR.

TABLE 1 Patient characteristics at study entry

Characteristic	
Patients – *N* (%)	399 (100.0)
Age	
Median (interquartile range)	57 (46–69)
Age group – *N* (%)	
16–29	34 (8.5)
30–39	39 (9.8)
40–49	48 (12.0)
50–59	94 (23.6)
60–69	92 (23.1)
70–79	69 (17.3)
>79	23 (5.8)
Sex – *N* (%)	
Male	231 (57.9)
Female	168 (42.1)
Gender/pregnancy – *N* (%)	
Male and female (nulliparous)	243 (60.9)
Female (≥1 pregnancy)	156 (30.1)
Weight – kg	
Median (interquartile range)	60 (53–66)
Height – cm	
Median (interquartile range)	165 (160–170)
Body mass index	
Median (interquartile range)	21.4 (19.6–24.1)
Body surface area – m^2	
Median (interquartile range)	1.6 (1.5–1.7)
Disease – *N* (%)	
Acute leukemia	126 (31.6)
Chronic leukemia	7 (1.8)
Multiple myeloma	27 (6.8)
Myelodysplastic syndrome	29 (7.3)
Lymphoma	83 (20.8)
Thrombocytopenia	50 (12.5)
Solid tumor	31 (7.8)
Other	46 (11.6)
Treatment – *N* (%)	
Chemotherapy or radiation	302 (75.7)
Other supportive care	97 (24.3)

2 | MATERIALS AND METHODS

2.1 | Patient inclusion criteria

The records of 399 patients who met the inclusion criteria between January 1, 2013, and December 31, 2017 were retrospectively reviewed in the hospital and blood bank databases. The detailed patient characteristics are shown in Table 1. The inclusion criteria were as follows: (1) nonsurgical patients who received at least two PLT transfusions and who had not received blood components before the first PLT transfusion during the course of study; and (2) available diagnostic and clinical information, including clinical characteristics, post-PLT transfusion increments (within 4 h and/or 18–24 h), PLT antibody screening, prior history of pregnancy or blood transfusion, which was available during the study period. The follow-up time was defined as the time elapsed from the first to last PLT transfusion. All patients were followed for the entire duration of their requirement for PLT transfusions during the study period. All patients were monitored clinically during random apheresis PLT transfusions. To

assess the relationship between Tregs and PTR, we prospectively and randomly selected 24 patients with hematologic disease (mean age, 51.2 years) and PTR to form the research group, including 12 patients with acute leukemia, 7 with lymphoma, and 5 with myelodysplastic syndromes. The control group comprised 33 patients with hematological disease and without PTR (mean age 52.5 years), including 17 patients with acute leukemia, 10 patients with lymphoma, and 6 patients with myelodysplastic syndrome. Approval was obtained from the review board of the Sir Run Run Shaw Hospital, Zhejiang University School of Medicine.

2.2 | Definitions of alloimmunization and refractoriness

Alloimmunization was defined as the development of PLT antibodies in patients previously negative for PLT antibodies during the study period. A low corrected count increment (CCI) was defined as CCI (≤4 h) <5 or CCI (18–24 h) <4.5. Platelet refractoriness was defined as the occurrence of a low CCI in two consecutive PLT transfusions. CCI was calculated as follows[11]:

$$\text{CCI} = \text{platelet increment}\ \left(10^9/\text{L}\right) \times \text{body surface area}\ \left(\text{m}^2\right)/\text{platelets transformed}\ (10^{11}).$$

Body surface area was calculated as follows: 0.0061 × height (cm) + 0.0128 × weight (kg)−0.1529.[14]

2.3 | PLT antibody assay

Patient phlebotomy time before PLT transfusion: Serum samples were obtained from clotted blood following centrifugation at 1000 ***g*** for 10 min. Commercially available monoclonal antibody solid-phase platelet antibody test (MASPAT) kits (Sanquin) were used to screen for platelet antibodies according to the manufacturer's guidelines. PLTs were screened using the following antigens: HPA-1(a, b); HPA-2(a, b); HPA-3(a, b); HPA-4(a); HPA-5(a, b); HLA-A1, 2, 3, 30, 31, 68; HLA-B7, 35, 38, 44, 51, 57, 60; and HLA-C3, 4.

2.4 | T cell and cytokine detection

Whole blood was used for the analyses of Tregs. Characterization of these cells was performed by flow cytometry using the following monoclonal antibodies: $CD3^-$ FITC, $CD4^-$ PE, $CD8^-$ APC-Cy7, $CD25^-$ APC, $CD127^-$ PE-Cy7, and mouse IgG1 APC and IgG1 PE-Cy7 as negative control (all from BD Biosciences). Tregs were characterized by $CD4^+CD25^+CD127^-$ expression. The results are expressed as percentages of the $CD4^+$ lymphocyte population. Plasma concentrations of TGF-β and IL-17 were detected by enzyme-linked immunosorbent assay according to the manufacturer's instructions (Tibikang Biological Technology Co. Ltd).

2.5 | Blood component preparation and transfusion

Blood components were prepared at the Blood Center of Zhejiang Province, China. All patients received apheresis PLTs; the upper limit of residual leukocytes was 5×10^8 white blood cells (WBCs)/bag, while the volume of apheresis PLTs was approximately 250–300 ml/bag. In our blood center, a single platelet unit contained ≥2.5×10^{11} platelets.

Packed red blood cells (RBCs) (non-leukoreduced) were administered at a hemoglobin level of <70 g/L. The PLT transfusion trigger was 20×10^9/L in patients receiving supportive therapy only (eg, for acute leukemia) and 30×10^9/L in other patients. All patients received ABO-compatible apheresis PLTs and were randomized to receive either a low dose (≤$2.5 \times 10^{11}/m^2$) or a high dose (>$2.5 \times 10^{11}/m^2$) of PLTs.

2.6 | Study endpoints

The primary endpoints were PTR and PLT antibody positivity before the diagnosis of refractoriness.

2.7 | Statistical analysis

Statistical significance was assessed using χ^2 tests. Statistical analysis was performed using SPSS for Windows, version 16.0. Statistical significance was considered at $p < 0.05$.

3 | RESULTS

3.1 | Patient enrollment and PLT transfusions

Between 2013 and 2017, 399 patients who met the inclusion criteria were retrospectively enrolled in this study. The median age of the patients was 57 years (interquartile range, 46–69 years). Among these non-transfused 399 patients, 156 had a history of pregnancy. The patients received a median of five PLT transfusions (interquartile range, 2–11) and three RBC transfusions (interquartile range, 1–7). The median duration of follow-up (from the first to last transfusion) was 20 weeks (interquartile range, 2–51) (Table 2).

3.2 | Alloimmunization and refractoriness

Of the 399 patients included in the study, 77 (19.3%) had refractoriness. Platelet antibodies were detected in 22 of these 77 patients (28.6%). Additionally, 35 of the 322 (10.9%) non-refractory patients were positive for PLT antibodies. Statistical analysis showed a significant increase in alloimmunization in the patients with PTR ($p < 0.001$) (Table 3).

TABLE 2 The effects of platelet transfusions

Characteristic	
Total platelet transfusions number – *N*	2946
Platelet transfusions (no. per patient)	
Median (interquartile range)	5 (2–11)
Platelet transfusions – 10^{11} (dose. per transfusion)	
Median (interquartile range)	4.3 (3.5–4.8)
Platelet transfusions – 10^{11} (dose. per square meter of body surface area)	
Median (interquartile range)	2.6 (2.1–3.0)
Duration of follow-up (weeks)	
Median (interquartile range)	20 (6–51)
Red-cell transfusions (no. per patient)	
Median (interquartile range)	3 (1–7)
Laboratory values and response to platelet transfusions	
Pretransfusion platelet count (10^9/L)	
No. with data	2677
Median (interquartile range)	14 (9–20)
Post-transfusion platelet count within 4 h (10^9/L)	
No. with data	1882
Median (interquartile range)	52 (34–72)
Platelet increment (≤4 h) (10^9/L)	
Median (interquartile range)	38 (19–56)
Post-transfusion CCI (≤4 h)	
No. of transfusions with all data available to calculate 4h CCI	1868
Median (interquartile range)	15.2 (8.0–21.5)
Post-transfusion platelet counts 18–24 h (10^9/L)	
No. with data	564
Median (interquartile range)	35 (18–55)
Platelet increment (18–24 h) (10^9/L)	
Median (interquartile range)	18 (2–36)
Post-transfusion CCI (18–24 h)	
No. of transfusions with all data available to calculate 18–24-h CCI	553
Median (interquartile range)	7.4 (0.9–14.5)
No. of transfusions with all data available to calculate CCI	2421

Before enrollment in this study, PLT antibodies were detected in 10 (2.5%) of the 399 patients included (Figure 1). One of these patients was male (0.4%), and nine were parous females (5.8%) ($p < 0.01$). The frequencies of alloimmunization during the study period were 11.5% in women and 11.9% in men and nulliparous women ($p = 0.905$). The number of alloimmunized patients was significantly higher in patients with hematologic disease than in the other disease groups ($p < 0.05$). Among 399 patients who received at least two PLT transfusions, PTR occurred in 77 patients (19.3%). The numbers of transfused blood components, diagnosis,

TABLE 3 Platelet antibody and platelet refractoriness

Platelet antibodies	Non- PTR patients *N* (%)	PTR patients *N* (%)	*p*
Negative patients (*n* = 342)	287 (89.1)	55 (71.4)	
Positive patients (*n* = 57)	35 (10.9)	22 (28.6)	<0.001
Total (*n* = 399)	322 (100.0)	77 (100.0)	

and frequencies of refractoriness and alloimmunization are shown in Table 4.

3.3 | Low CCIs and individual transfusions

A total of 399 patients received 2946 transfusions, of which 1868 had acceptable CCI 4 h values and 553 had acceptable CCI 24 h values. As shown in Table 5, among the 57 antibody-positive patients who received 492 transfusions, 161 (32.7%) had low CCI. Regardless of the alloimmunization status, low CCIs were observed following 536 (22.1%) platelet transfusions. Low CCIs occurred significantly more frequently in parous women than in men and nulliparous women ($p = 0.017$). Of the 2087 subsequent transfusions, 484 (23.2%) had low CCIs. Low CCIs were more common in subsequent transfusions than in first transfusions (15.6%) ($p = 0.002$). However, no statistically significant differences were found in the occurrence of low CCI between low doses (≤$2.5 \times 10^{11}/m^2$) and high doses (>$2.5 \times 10^{11}/m^2$). Histograms of the distributions of CCI (<4 h) for these transfusions are shown in Figure 2.

3.4 | T lymphocyte subsets and related cytokines

No significant difference in the percentages of peripheral CD4, CD8, and $CD4^+CD25^+CD127^-$ Treg cells was observed between the PTR and control groups. No correlation was found between CD4/CD8, TGF-β1, and IL-17 levels in the peripheral circulation and PTR (Table 6).

4 | DISCUSSION

The incidence of alloimmunization has been estimated to be as high as 20%–60% among hematological patients globally requiring chronic PLT transfusion support.[15-17] The present study evaluated data from 399 patients who received over 2900 PLT transfusions and were followed for a median of 20 weeks. Pretransfusion antibody data were available for all patients who were enrolled in this study. The positivity rate in this study was lower than that reported in other studies in the Chinese population (5.8 vs. 24.7% in parous women).[18] There are several explanations for the lower prevalence of PLT antibodies in our study. First, the median age of patients in

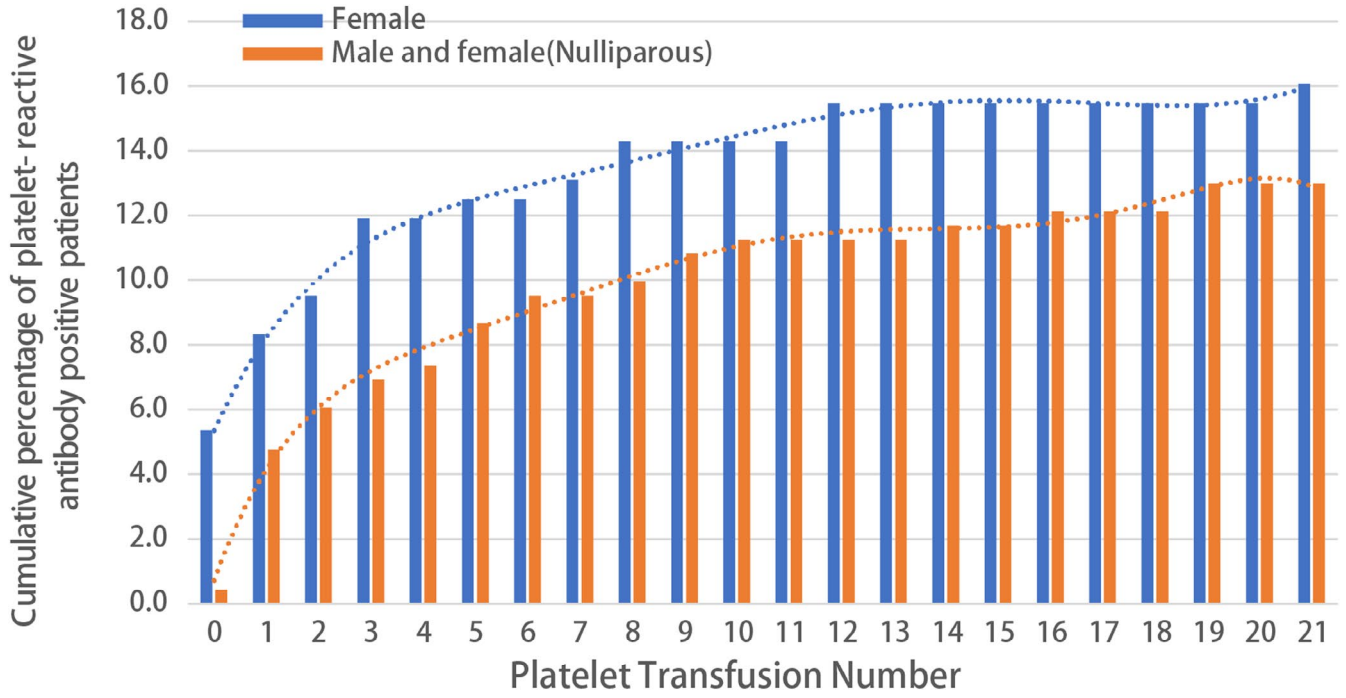

FIGURE 1 Proportions of female and male patients with PLT antibodies in relation to the numbers of PLT transfusions

TABLE 4 Alloimmunization and platelet refractoriness

			Antibodies positive patients			
	Patients *N*	Mean number of PLT transfusions *N*	Before study entry *N* (%)	Newly generated *N* (%)	Total number *N* (%)	Refractoriness patients *N* (%)
Totals	399	6.1	10 (2.5)	47 (11.8)	57 (14.3)	77 (19.3)
Gender/pregnancy						
Male and nulliparous female	243	5.9	1 (0.4)[a]	29 (11.9)	30 (12.3)	47 (19.3)
Female (≥1 pregnancy)	156	6.3	9 (5.8)	18 (11.5)	27 (17.3)	30 (19.2)
Age group						
16–29	34	5.4	0 (0.0)	4 (11.8)	4 (11.8)	6 (17.7)
30–39	39	6.3	0 (0.0)	4 (10.3)	4 (10.3)	11 (28.2)
40–49	48	6.4	2 (4.2)	7 (14.6)	9 (18.8)	10 (20.8)
50–59	94	6.3	0 (0.0)	8 (8.5)	8 (8.5)	12 (12.8)
60–69	92	5.6	3 (3.3)	11 (11.9)	14 (15.2)	19 (20.7)
70–79	69	6.5	4 (5.8)	12 (17.4)	16 (23.2)	14 (20.3)
>79	23	5.3	1 (4.3)	1 (4.3)	2 (8.7)	5 (21.7)
Disease						
Hematologic disease	341	8.1	10 (2.9)	46 (13.5)[b]	56 (16.4)[b]	68 (19.9)
Other disease	58	3.0	0 (0.0)	1 (1.7)	1 (1.7)	9 (15.5)

[a]There are significant differences between Male\ nulliparous Female groups and Female groups, chi-square test, $p < 0.05$.

[b]There are significant differences between hematologic disease groups and other disease groups, chi-square test, $p < 0.05$.

our study was relatively high, at 57 years, while the donors from the blood center were generally young (data not provided). Although the proportion of women with a history of pregnancy was similar, detectable HLA antibodies in pregnant women decreased over time.[19] Secondly, the Luminex platform may be more sensitive than the MASPAT kits used in our study. Furthermore, up to 4.63% males and 4.90% nulliparous female donors showed positive reactions in the HLA antibody screening test in that study, while our data showed a low prevalence of PLT antibodies in men and nulliparous women. The cause of the low level of antibodies in non-transfused males and non-transfused, nulliparous women is unknown and needs further studies.

All patients in our study received apheresis PLT, and 11.8% of the patients became transiently antibody-positive during the study period (Table 4). The incidence of antibody positivity in our study was higher than that in the PLADO trial, in which approximately 5% of patients became alloimmunized within 30 days of the trial,[11] and lower than that in the TRAP trial, which reported an alloimmunization rate of at least 18%.[9] The following factors could explain these differences in results. First, the median follow-up time in our study was 20 weeks, which was longer than that in the PLADO trial. Second, the upper limit of residual leukocytes was 5×10^8 WBCs/bag of apheresis PLTs in our study. The presence of "passenger" leukocytes enhances the immunogenicity of non-leukoreduced apheresis PLTs in transfusion recipients, making the patients more susceptible to developing alloantibodies. All study patients in the PLADO trial received leukoreduced PLTs, which may explain the lower incidence of alloimmunization in this trial. Furthermore, the prophylactic PLT transfusion trigger threshold (20×10^9/L) at our institution is higher than that commonly used in Western countries (10×10^9/L). This will lead to an increased number of PLT transfusions per patient which in turn may increase the incidence of both alloimmunization and PTR. Finally, as pregnancy can also induce alloimmunization,[20] it should also be mentioned that 93% of the women in the current study had previously been pregnant as compared with 68% in the PLADO and 81% in TRAP studies. The proportion of newly produced antibodies in males and non-parous females (11.9%) was almost identical to that in previously pregnant females (11.5%) during the study period (Figure 1 and Table 4). This observation highlights the importance of PLT transfusion as a major alloimmunization factor in multiply transfusion patients.

Refractoriness, defined as two consecutives low CCI measures, was common, occurring in 19.3% of all patients, with no significant differences between sexes or among age groups and diseases ($p > 0.05$). The observed refractoriness rates were higher than those reported in the PLADO study (14%), perhaps because of the longer

TABLE 5 Platelet and patient characteristics associated with the occurrence of low CCI

	Total number of CCIs	Low CCIs, *N* (%)
Total	2421	536 (22.1)
Platelet antibodies		
Positive patients	492	161 (32.7)[a]
Negative patients	1929	375 (19.4)
Gender/pregnancy		
Male and female (nulliparous)	1445	296 (20.5)[a]
Female (≥1 pregnancy)	976	240 (24.6)
Treatment dose		
Low dose (≤2.5×10^{11}/m^2)	1096	245 (22.4)
High dose (>2.5×10^{11}/m^2)	1325	291 (22.0)
Platelet transfusion number		
First transfusion	334	52 (15.6)[a]
Subsequent transfusion	2087	484 (23.2)
Red-cell transfusion number		
0 transfusion	451	96 (21.3)
≥1 transfusion	1970	430 (21.8)

[a]Chi-square test, $p < 0.05$.

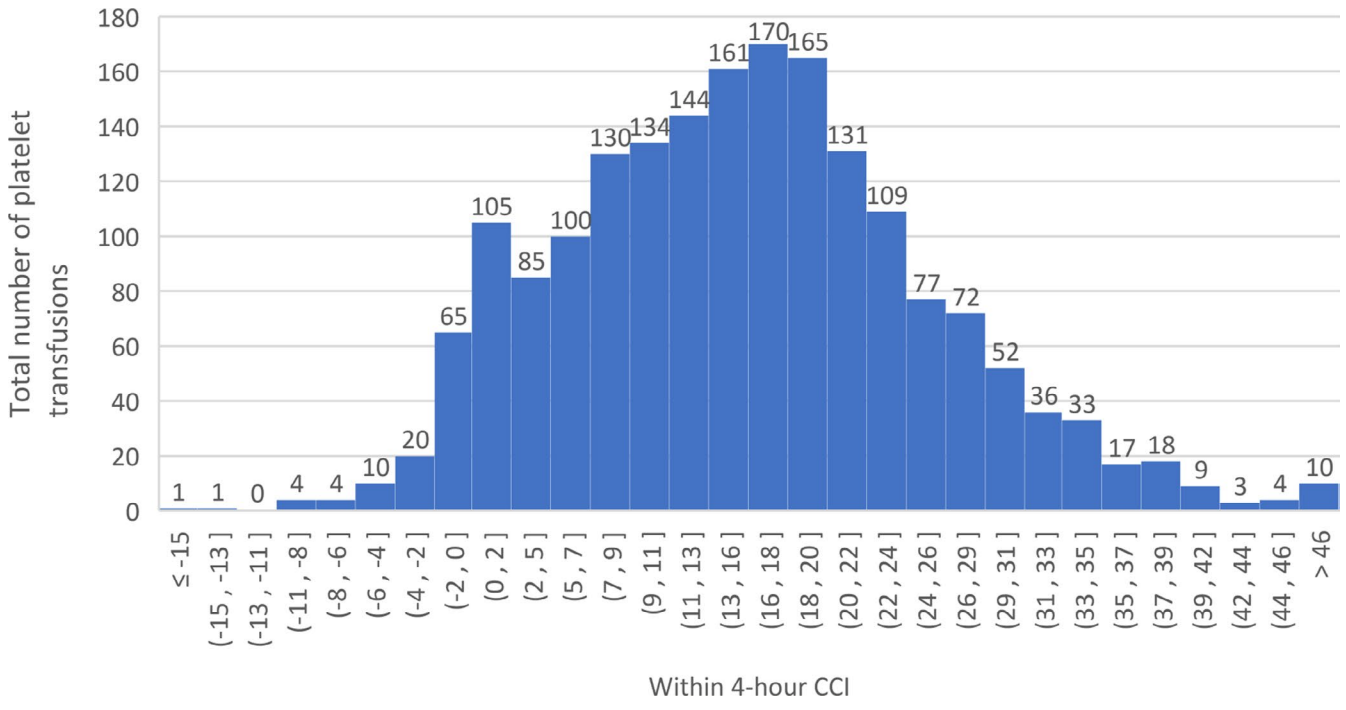

FIGURE 2 Distribution of CCI 4h

TABLE 6 Comparison of Tregs and related cytokines in peripheral blood of PTR and control group

	N	CD4 (%)	CD8 (%)	CD4/CD8	Tregs (% of CD4)	TGF-β1 (pg/ml)	IL-17 (pg/ml)
PTR	24	36.51 (±12.12)	55.61 (±12.34)	0.74 (±0.40)	3.28 (±2.42)	972.06 (±785.74)	4.74 (±0.97)
Control	33	32.33 (±15.07)	59.82 (±15.83)	0.66 (±.53)	2.09 (±1.95)	1488.02 (±1253.01)	5.06 (±1.88)
p		0.415	0.341	0.847	0.30	0.068	0.735

follow-up period. In this study, 22.1% of all transfusions with sufficient data for calculation had low CCIs. As shown in Table 5, lower CCI measurements were more common in subsequent PLT transfusions, alloimmunized patients, and women with ≥1 pregnancy than in patients undergoing their first PLT transfusion, platelet antibody-negative patients, and males. There are at least 3 reasons why lower CCIs were more common in subsequent PLT transfusions than in the first transfusion: first, only patients with an inadequate response require further transfusions; second, multiple PLT transfusions increase the likelihood of PLT immunization; third, patients who continue to receive PLT transfusions tend to have a longer duration of illness and are more likely to develop other factors that lead to low CCIs. Similar effects were observed in the analysis of the PLADO trial.[10,11] However, no significant associations were observed between low CCI and the treatment dose in this study. In the PLADO trial, the low-dose treatment group was more likely to have at least one CCI < 5000 (40%) than the medium-dose (21%) and high-dose (17%) groups.[11] This result may be related to differences in dose grouping, which requires further assessment. Moreover, no significant associations were found between low CCI and red cell transfusion number ($p = 0.801$).

$CD4^+CD25^+$ Treg cells are natural Tregs that have been extensively studied for their role in autoimmune diseases, transplant immune tolerance, and maintenance of immune balance.[21,22] We investigated the associations of Treg, TGF-β1, and IL-17 levels in the peripheral blood with PTR by comparing the levels of $CD4^+CD25^+CD127^-$ Tregs and related cytokines between the PTR and control groups, but found no significant difference between the patients and controls (Table 6).

The timing of sample collection differed among patients, which may have limited our analysis of the kinetics of the alloimmunization response. Moreover, some individual responses may have been missed due to the timing of sample collection, although we would not expect this to differ between groups. Additionally, the MASPAT method cannot identify the specificity of platelet antibodies.

In summary, we systematically analyzed here, the development of PLT antibodies in patients receiving multiple transfusions. We found that refractoriness was common in these patients (19.3%) and that alloimmunization was present in 28.6% of the patients, suggesting that alloimmunization is one of the most important causes of refractoriness. Our findings also suggest that monitoring the proportion of Tregs in peripheral blood has little significance in predicting PTR.

5 | INFORMED CONSENT

All recruited patients provided written informed consent prior to data collection.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ETHICAL APPROVAL

This study was approved by the Institutional Review Board of Sir Run Run Shaw Hospital School of Medicine, Zhejiang University.

DATA AVAILABILITY STATEMENT

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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How to cite this article: Song T, Zhang Y, Huang J, Liu Z. Transfusion-induced platelet antibodies and regulatory T cells in multiply transfused patients. *J Clin Lab Anal*. 2021;35:e23864. https://doi.org/10.1002/jcla.23864