STIMULUS REPORT

blood advances

Whole-blood phenotyping to assess alloimmunization status in transfused sickle cell disease patients

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Key Points

- CXCR5, PD1, and ICOS markers expressed on $CD4^+$ TLs can differentiate between responder and nonresponder alloimmunization statuses.
- Quiescent Tfh cells were less frequent in the blood of nonalloimmunized polytransfused patients.

It is essential to limit hemolytic transfusion reactions in polytransfused individuals, and the prevention of alloimmunization is a key solution. $CD4^+$ T lymphocyte (TL) markers, particularly follicular T helper (Tfh) cells, may differentiate between responder and nonresponder alloimmunization statuses. We tested this hypothesis by studying the phenotype of $CXCR5^+PD1^+$ TLs in whole blood. Our results suggest that high levels of $CXCR5^+PD1^+CD4^+$ TLs in whole blood may be a characteristic of nonalloimmunized patients. However, these cells did not display the phenotypic characteristics of active Tfh cells. Instead, a decrease in blood quiescent Tfh-cell levels was observed in nonalloimmunized polytransfused patients. High levels of $CXCR5^+PD1^+CD4^+$ TLs may be associated with inhibitory signaling functions of T cells, as reflected by the low levels of $PD1^+ICOS^+$ cells in the nonalloimmunized polytransfused group. The description of these particular phenotypes, and their comparison among groups of patients, responders, and nonresponders, suggests that new immunological components should be considered when trying to understand posttransfusion alloimmunization.

Introduction

The risk of posttransfusion alloimmunization is a major problem in polytransfused patients.[1] Despite extensive research, the mechanisms underlying the strong responses in some patients, but not others, remain unknown.[2] Studies in mouse models have shown that alloimmunization has the features of classical immunization, and agonists of innate immunity remain the best way to induce alloimmunization.[3-7] These agonists include poly(I:C) in particular, which is known to be efficient in vaccination.[8] However, this relationship between innate immunity and adaptative immune responses has never been observed for red blood cell (RBC) alloimmunization in humans. Moreover, other immunological, genetic, or epigenetic elements may be involved.

Alloimmunization primarily involves the production of antibodies directed against blood products, and $CD4^+$ T lymphocytes (TLs) are another pillar of alloimmunization responses.[9,10] We have shown that several $CD4^+$ TL markers can differentiate between responder and nonresponder statuses.[11] In particular, we surprisingly found that nonalloimmunized patients had higher levels of $CD4^+CXCR5^+PD1^+$ TLs in whole blood than healthy donors (HDs) or alloimmunized patients (see Figure 6B in Vingert et al[11]). This phenotype, which is usually associated with follicular T helper (Tfh) differentiation,[12,13] was unexpected in a group of nonalloimmunized patients. It was all the more surprising given that we found circulating antigen-specific and functional Tfh cells in alloimmunized patients, but not in nonalloimmunized patients.[11] Other groups have also suggested a possible role for Tfh cells in alloimminization.[14,15]

Submitted 5 October 2020; accepted 22 January 2021; published online 2 March 2021. DOI 10.1182/bloodadvances.2020003537.

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The full-text version of this article contains a data supplement.

Table 1. Characteristics of study subjects

	SCD patients		
	Alloimmunized (n = 14)	Nonalloimmunized (n = 9)	HDs (n = 10)
Age, y	37.2 (19-61)	39 (21-49)	32.5 (32.5-50)
Sex ratio (male/female)	0.75	0.8	1.5
Leukoreduced units (sampling time)	11.5 (3-45)	241 (148-598)	—
Leukoreduced units (alloimmunization time)	6.5 (2-42)	—	—

Median values (range) are reported.

It has recently been reported that $CD4^+$ T cells from alloimmunized patients may differentiate more easily into Tfh cells in vitro.[16]

It is difficult to study alloimmunization at the precise time at which it occurs in patients. Observation of the $CXCR5^+PD1^+CD4^+$ T-cell subset might make it easier to distinguish between polytransfused patients earlier. This early distinction could potentially reduce the risk of alloimmunization and make transfusion more efficient. We tested this hypothesis by recruiting 2 groups of patients of known alloimmunization response (already shown to display or not display alloimmunization). We studied polytransfused sickle cell disease (SCD) patients, extensively exploring the phenotype of the $CXCR5^+PD1^+$ cells from the whole blood of these patients, to determine whether they included a circulating Tfh subpopulation or whether a more precise phenotype could be identified for differentiation between these 2 groups of polytransfused patients. The use of whole blood was essential for this approach, because separation procedures are known to alter chemokine receptor expression.[17-19]

The results of this study are potentially relevant for increasing the efficiency of transfusion and reducing the frequency of alloimmunization.

Methods

Patients and recruitment

Two groups of polytransfused adult SCD patients were included in this study. Alloimmunized (n = 14) and nonalloimmunized (n = 9) patients were recruited from the adult SCD referral center at Henri Mondor Hospital (Créteil, France). The alloimmunized patients were considered to be strong responders, because alloimmunization occurred after the transfusion of a median of only 11.5 RBC units. The nonalloimmunized patients were considered to be weak responders, as immunization had not occurred despite transfusion with a median of 241 RBC units per patient. The control group consisted of ethnically matched healthy blood donors (HD group, n = 10) supplied by the Etablissement Français du Sang (Table 1). The study was approved by the CPP IDF IX (Medical Ethics Committee) under agreement number 10-040. The participating patients or HDs had not had any infection (viral, bacterial, fungal, or yeast) or been vaccinated in the 30 days preceding inclusion, and all gave written informed consent.

Whole-blood phenotyping

Fresh whole-blood lymphocytes were used for $CD4^+$ T-cell phenotyping without a separation procedure, as previously described, because separation alters chemokine receptor expression. $CD4^+$ T-cell phenotyping was performed within 2 hours of sampling.[11] We distinguished T-cell subsets on the basis of their CD45RA expression, and the $CD45RA^-$ subset was analyzed.

Leukocytes were labeled with the following antibodies: anti-CD4 PE-CF594, anti-CD3 AF700, anti-CD45RA APC-H7, anti-CXCR5 AF488, anti-CCR6 PE-Cy7, anti-CXCR3 PE-Cy5, anti-CD279 PE (BD Biosciences, San Jose, CA), and anti-CD278 BV421 (BioLegend, San Diego, CA). Aqua LIVE/DEAD viability dye (Thermo Fisher Scientific, Waltham, MA) was added to exclude dead cells. Fluorescence was assessed with a 20-parameter LSR Fortessa flow cytometer (BD Biosciences). The performance of the flow cytometer was checked before each assay. Data were analyzed with FlowJo software (v10.1r5; Ashland, OR).

Statistical analysis

All analyses were performed with Prism 6.07 software (GraphPad Software, La Jolla, CA). All significant differences between groups ($P < .05$) are indicated on the data plots.

Results and discussion

We studied the phenotype of $CXCR5^+PD1^+CD4^+$ T cells from SCD patients in detail, all analyses being performed exclusively on whole blood. For alloimmunized SCD patients, the observed immunophenotypic expression may actually be a result rather than a cause of allosensitization events. No recent immune activation (CD38, HLA-DR, CD154, or CD69) or differences were detected in these 2 groups of patients or in comparisons of these patients with the HD group (data not shown). In addition, as indicated in "Methods," the patients were not suffering from any viral, bacterial, fungal, or bacterial infections and had not been vaccinated in the last 30 days.

We first determined the levels of $CXCR5^+PD1^+$ cells among live and activated $CD4^+$ T cells (Aqua $LIVE/DEAD^-CD45RA^-$) (Figure 1A). As in our previous study,[11] performed under the same experimental conditions, $CXCR5^+PD1^+$ cells were significantly more frequent in nonalloimmunzed patients than in alloimmunized patients (Figure 1B; 13.10 ± 3.64% vs 5.19 ± 3.12%, $P < .005$) and HDs ($P < .0001$).

These results conflict with those of Balbuena-Merla et al.[16] The difference between these studies may reflect differences in recruitment or transfusion practices. We found no significant differences in the number of alloantibodies per responder patient (supplemental Figure 1A). However, in France, patients receive Kell pheno-compatible blood, and our responder patients have no anti-K response (supplemental Figure 1B). Conversely, we have many ethnically discordant transfusions. We therefore observe high rates of anti-Jkb responses (supplemental Figure 1B). The observed

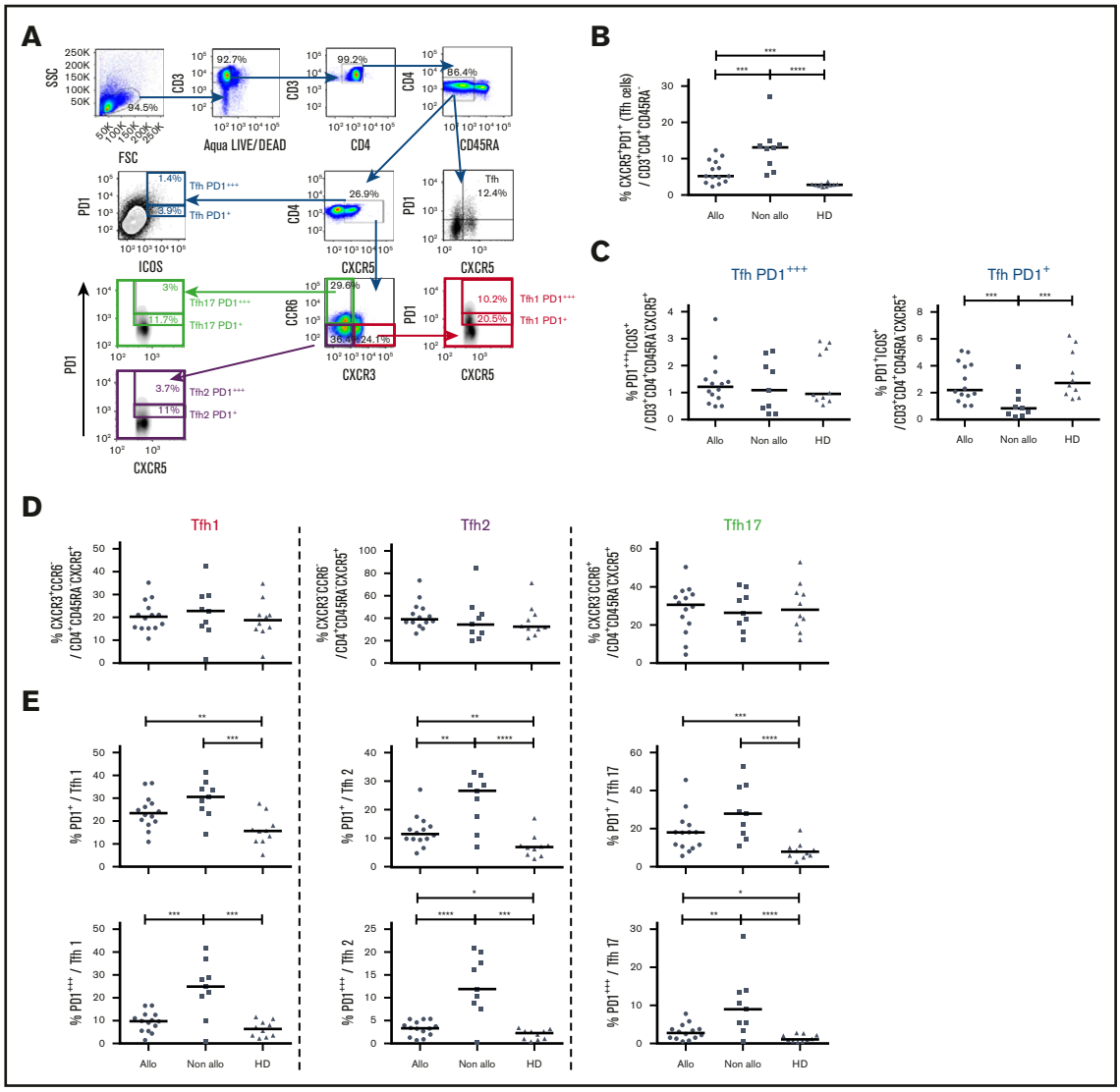

Figure 1. Comparison of the phenotype of Tfh subpopulations in whole-blood lymphocytes from alloimmunized patients, nonalloimmunized patients, and HDs. (A) Gating strategy for flow cytometry analysis. Lymphocytes were gated on size (forward scatter [FSC]) and granularity (side scatter [SSC]). Dead cells were excluded with Aqua LIVE/DEAD staining. Analyses were performed on $CD4^+CD45RA^-$ cells. The Tfh population was identified with the $CXCR5^+PD1^+$ gate. The Tfh $PD1^{+++}$ and Tfh $PD1^+$ populations were identified with the $CD4^+CD45RA^-CXCR5^+ICOS^+PD1^{+++}$ and $CD4^+CD45RA^-CXCR5^+ICOS^+PD1^+$ gates, respectively. The Tfh1, Tfh2, and Tfh17 populations were identified with the $CD4^+CD45RA^-CXCR5^+CCR6^-CXCR3^+$, $CD4^+CD45RA^-CXCR5^+CCR6^-CXCR3^-$, and $CD4^+CD45RA^-CXCR5^+CCR6^+CXCR3^-$ gates, respectively. (B) Comparison of Tfh percentages in whole blood between alloimmunized (n = 14, black circles, 14 experiments, with 1 donor per experiment) and nonalloimmunized (n = 9, black squares, 9 experiments, with 1 donor per experiment) transfused SCD patients. Ethnically matched HDs (n = 10, black triangles, 10 experiments, with 1 donor per experiment) were used as a control group. (C) Comparison of PD1 expression on the Tfh $CXCR5^+ICOS^+$ subpopulation in whole-blood lymphocytes from patients and HDs. (D) Comparison of CXCR3 and CCR6 expression between Tfh subsets in whole-blood lymphocytes from patients and HDs. (E) Comparison of the $PD1^+$ and $PD1^{+++}$ subsets in the Tfh1, Tfh2, and Tfh17 subpopulations in whole-blood lymphocytes from patients and HDs. Horizontal bars indicate the median values. Significant *P* values (< .05) were obtained in analysis of variance and post hoc tests. $^*P < .05$; $^{**}P < .01$; $^{***}P < .005$; $^{****}P < .001$.

differences between studies may also be due to the use of Ficoll for the isolation of $CXCR5^+PD1^+CD4^+$ T cells. Differences in chemokine receptor expression depend on the exploration method used.[17-19] Our results confirmed differences in expression between Ficoll isolation vs whole-blood methods for CXCR5, CCR6, and CXCR3 expression (supplemental Figure 2). These differences may also result from the analysis, which may be based on a logarithmic scale and rectangular gates or a biexponential scale and spider gates as used by Balbuena-Merla et al.[16]

High levels of $CXCR5^+PD1^+CD4^+$ T cells in whole blood may be a characteristic of nonalloimmunized patients, but it remains unclear if they are of any functional benefit. A functional study of these $CXCR5^+PD1^+$ cells revealed no significant differences between patient groups (supplemental Figure 3).

Circulating active Tfh cells are much less frequent in blood than in secondary lymphoid organs.[20] We studied their presence in the blood of SCD patients by evaluating PD1 expression in $CXCR5^+$

$ICOS^+$ cells (Figure 1A). We detected no active $PD1^{+++}$ Tfh cells in the 2 groups of patients, and the levels of these cells were very similar to those in HDs (Figure 1C, left panel). The high levels of $CXCR5^+PD1^+CD4^+$ T cells were, therefore, highly unlikely to be related to the presence of Tfh cells. In addition, the number of quiescent Tfh cells, $CXCR5^+ICOS^+PD1^+CD4^+$ T cells,[20] was significantly lower in the nonalloimmunized group than in the alloimmunized or HD group ($P < .005$) (Figure 1C, right panel). These low levels of blood quiescent $PD1^+$ Tfh cells in nonalloimmunized patients may play a role in their status as nonresponders to alloimmunization.

Circulating Tfh cells can also be divided into Tfh1, Tfh2, and Tfh17 subsets on the basis of their ability to express the CXCR3 and CCR6 chemokine receptors.[21] We therefore evaluated these subsets to determine the origin of the $CXCR5^+PD1^+$ cells in the nonresponder group (Figure 1A). These subpopulations were present at similar levels in all patients, regardless of their alloimmunization status, and in the HD group (Figure 1D). The levels detected differed from those in another recently published work,[16] again due to the use of a different lymphocyte isolation procedure, but we can confirm the absence of a significant difference.

By contrast, PD1 expression distinguished between nonresponders and responders in a highly significant manner, regardless of the levels of Tfh subsets (Figure 1A). As in $CD4^+$ TLs, PD1 levels were significantly higher in the nonresponder group for all Tfh subsets (Figure 1E). PD1 is associated principally with the inhibitory signaling functions of T cells. It was, therefore, probably this function that we were observing in nonresponders rather than an increase in the levels of Tfh cells. This hypothesis was also supported by the lower levels of $PD1^+ICOS^+$ cells (Figure 1C, right panel). ICOS is a costimulatory signal for T-cell activation. The lower levels of this marker in nonresponders are consistent with a weaker alloimmunization status.

The prevention of alloimmunization, improvements in transfusion performance, and the limitation of hemolytic transfusion reactions are essential for polytransfused individuals.[22] Our data may be relevant to alloimmunization status, particularly for SCD patients. PD1 expression by $CD4^+$ T cells should be considered in addition to the recommendations already described.[23]

Acknowledgments

The authors are particularly grateful to the patients who participated in this study.

This work was supported by the Etablissement Français du Sang, INSERM, and Université Paris-Est.

Authorship

Contribution: B.V. was the principal investigator and takes primary responsibility for this paper; P.B., A.H., and F.G. recruited patients; S.P. was the clinical research associate for this study; M.T. performed the laboratory work; M.T. and B.V. analyzed the results; B.V. and F.P. coordinated the research; and M.T., B.V., and F.P. wrote the paper.

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

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