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 immune and adaptive 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 al11). 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 alloimmunization.14,15
It has recently been reported that CD4⁺ T cells from alloimmunized patients may differentiate more easily into Tfh cells in vitro. 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. 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. 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, performed under the same experimental conditions, CXCR5⁺PD1⁺ cells were significantly more frequent in nonalloimmunized 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. 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
Our results confirmed differences in expression between PD1 and PD1+ HDs. 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-CXCR5+ 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. Ethically 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.

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. We studied their presence in the blood of SCD patients by evaluating PD1 expression in CXCR5+...
ICOS<sup>+</sup> cells (Figure 1A). We detected no active PD1<sup>++</sup> 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<sup>+</sup>CD4<sup>+</sup> T cells were, therefore, highly unlikely to be related to the presence of Tfh cells. In addition, the number of quiescent Tfh cells, CXCR5<sup>+</sup>ICOS<sup>+</sup>PD1<sup>+</sup>CD4<sup>+</sup> T cells, 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<sup>+</sup> 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. We therefore evaluated these subsets to determine the origin of the CXCR5<sup>+</sup>PD1<sup>+</sup> 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, 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<sup>+</sup> 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<sup>+</sup>ICOS<sup>+</sup> 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. Our data may be relevant to alloimmunization status, particularly for SCD patients. PD1 expression by CD4<sup>+</sup> T cells should be considered in addition to the recommendations already described.

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**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.

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**References**

1. Yazdanbakhsh K, Ware RE, Noizat-Pirenne F. Red blood cell alloimmunization in sickle cell disease: pathophysiology, risk factors, and transfusion management. Blood. 2012;120(3):528-537.

2. Hudson KE, Fasano RM, Karafin MS, Hendrickson JE, Francis RO. Mechanisms of alloimmunization in sickle cell disease. Curr Opin Hematol. 2019;26(6):434-441.

3. Yu J, Heck S, Yazdanbakhsh K. Prevention of red cell alloimmunization by CD25 regulatory T cells in mouse models. Am J Hematol. 2007;82(8):691-696.

4. Hendrickson JE, Roback JD, Hillyer CD, Easley KA, Zimring JC. Discrete Toll-like receptor agonists have differential effects on alloimmunization to transfused red blood cells. Transfusion. 2008;48(9):1869-1877.

5. Gibb DR, Liu J, Natarajan P, et al. Type I IFN is necessary and sufficient for inflammation-induced red blood cell alloimmunization in mice. J Immunol. 2017;199(3):1041-1050.

6. Liu D, Gibb DR, Escamilla-Rivera V, et al. Type I IFN signaling critically regulates influenza-induced alloimmunization to transfused KEL RBCs in a murine model. Transfusion. 2019;59(10):3243-3252.

7. Elayeb R, Tamagne M, Bierling P, Noizat-Pirenne F, Vingert B. Red blood cell alloimmunization is influenced by the delay between Toll-like receptor agonist injection and transfusion. Haematologica. 2016;101(2):209-218.

8. Martins KA, Bavari S, Salazar AM. Vaccine adjuvant uses of poly-IC and derivatives. Expert Rev Vaccines. 2015;14(3):447-459.

9. Hendrickson JE, Saakadze N, Cadwell CM, et al. The spleen plays a central role in primary humoral alloimmunization to transfused mHEL red blood cells. Transfusion. 2009;49(8):1678-1684.

10. Natarajan P, Liu D, Patel SR, et al. CD4 depletion or CD40L blockade results in antigen-specific tolerance in a red blood cell alloimmunization model. Front Immunol. 2017;8:907.
11. Vingert B, Tamagne M, Habibi A, et al. Phenotypic differences of CD4(+) T cells in response to red blood cell immunization in transfused sickle cell disease patients. *Eur J Immunol.* 2015;45(6):1868-1879.

12. Crotty S. T follicular helper cell differentiation, function, and roles in disease. *Immunity.* 2014;41(4):529-542.

13. Ueno H, Banchereau J, Vinuesa CG. Pathophysiology of T follicular helper cells in humans and mice. *Nat Immunol.* 2015;16(2):142-152.

14. Godefroy E, Zhong H, Pham P, Friedman D, Yazdanbakhsh K. TIGIT-positive circulating follicular helper T cells display robust B-cell help functions: potential role in sickle cell alloimmunization. *Haematologica.* 2015;100(11):1415-1425.

15. Arneja A, Salazar JE, Jiang W, Hendrickson JE, Zimring JC, Luckey CJ. Interleukin-6 receptor-alpha signaling drives anti-RBC alloantibody production and T-follicular helper cell differentiation in a murine model of red blood cell alloimmunization. *Haematologica.* 2016;101(11):e440-e444.

16. Balbuena-Merle R, Santhanakrishnan M, Devine L, et al. Characterization of circulating and cultured Tfh-like cells in sickle cell disease in relation to red blood cell alloimmunization status. *Transfus Apher Sci.* 2020;102778.

17. Berhanu D, Mortari F, De Rosa SC, Roederer M. Optimized lymphocyte isolation methods for analysis of chemokine receptor expression. *J Immunol Methods.* 2003;279(1-2):199-207.

18. Lee B, Sharron M, Montaner LJ, Weissman D, Doms RW. Quantification of CD4, CCR5, and CXCR4 levels on lymphocyte subsets, dendritic cells, and differentially conditioned monocyte-derived macrophages. *Proc Natl Acad Sci USA.* 1999;96(9):5215-5220.

19. Lee B, Doranz BJ, Rana S, et al. Influence of the CCR2-V64I polymorphism on human immunodeficiency virus type 1 coreceptor activity and on chemokine receptor function of CCR2b, CCR3, CCR5, and CXCR4. *J Virol.* 1998;72(9):7450-7458.

20. Locci M, Havenar-Daughton C, Landais E, et al; International AIDS Vaccine Initiative Protocol C Principal Investigators. Human circulating PD-1(+)CXCR3 CXCR5(+) memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. *Immunity.* 2013;39(4):758-769.

21. Morita R, Schmitt N, Bentebibel SE, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity.* 2011;34(1):108-121.

22. Thein SL, Pirenne F, Fasano RM, et al. Hemolytic transfusion reactions in sickle cell disease: underappreciated and potentially fatal. *Haematologica.* 2020;105(3):539-544.

23. Chou ST, Alsawas M, Fasano RM, et al. American Society of Hematology 2020 guidelines for sickle cell disease: transfusion support. *Blood Adv.* 2020;4(2):327-355.