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Red Blood Cell Transfusions Are Associated with HLA Class I but not H-Y Alloantibodies in Children with Sickle Cell Disease

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Master of Science

Clinical Research

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An abstract of

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ABSTRACT

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By Robert S. Nickel, M.D.

Blood transfusions can induce alloantibodies to antigens on red blood cells (RBCs), white blood cells, and platelets, with these alloantibodies having importance in transfusion and transplantation. While transfusion-related alloimmunization against RBC antigens and human leukocyte antigens (HLA) have been studied, transfusion-related alloimmunization to minor histocompatibility antigens (mHA) such as H-Y antigens has not been clinically characterized. We thus conducted a cross-sectional study of 114 children with sickle cell disease (SCD) and measured antibodies to 5 H-Y antigens and to HLA class I and class II. Few patients had H-Y antibodies, with no significant differences in the prevalence of any H-Y antibody observed among transfused females (7%), transfused males (6%), and never transfused females (4%). In contrast, HLA class I, but not HLA class II, antibodies were more prevalent among transfused than never transfused patients (class I: 33% vs 13%, p=0.046; class II: 7% vs 8%, p=0.67). After adjustment for age, splenectomy, and hydroxyurea this association between RBC transfusion and HLA class I alloimmunization remained significant.(p=0.042). Among transfused patients, RBC alloantibody history but not the amount of RBC transfusion exposure was associated with a high (>25%) HLA class I panel reactive antibody (PRA) on both univariate (OR 6.8, 95% CI 2.1-22.3) and multivariable analysis (OR 6.3, 95% CI 1.7-22.6). These results are consistent with immunologic responder and non-responder phenotypes, wherein a subset of patients with SCD may be at higher risk for transfusion-related alloimmunization.

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INTRODUCTION

Sickle cell disease (SCD) is an inherited blood disorder that causes serious morbidity and early mortality even with current supportive care (1-3). Hematopoietic stem cell transplantation (HSCT) is the only cure for SCD and can achieve excellent long-term results (4), however, a major problem with HSCT, especially for SCD, is graft rejection. The etiology of this graft rejection is multifactorial, but pre-transplant blood transfusions are believed to contribute by immunizing patients to donor histocompatibility antigens. Thus, the proposed casual mechanism is that pre-HSCT transfusions cause alloimmunization to donor histocompatibility antigens which then cause HSCT rejection. This research project focuses on issues related to the first part of this pathway (pre-HSCT transfusions → alloimmunization to donor histocompatibility antigens).

While previous work has already provided evidence that red blood cell (RBC) transfusions can cause human leukocyte antigen (HLA) alloimmunization (5-7), no clinical study has investigated transfusions' capacity to cause alloimmunization to minor histocompatibility antigens (mHA). We thus designed this research project to primarily investigate if RBC transfusions given in current clinical practice cause mHA alloimmunization. This area of study is important because experiments in animals have implicated transfusion-related mHA alloimmunization in inducing HSCT rejection (8-13).

To study mHA alloimmunization, we used an assay to detect antibodies to H-Y antigens, a well characterized group of mHA. Since H-Y antigens are expressed only in males, only females were thought to be at risk for H-Y sensitization and H-Y antibody development. We conducted a cross-sectional study of the prevalence of H-Y antibodies in transfused female, transfused male, and never transfused female SCD patients. Our primary hypothesis was that the transfused female group would uniquely have an increased proportion of patients with any H-Y antibodies.

Through this study we also sought to better characterize transfusion-related HLA alloimmunization. First, we sought to validate other studies' findings performed in different patient populations (5-7) that suggest RBC transfusions cause HLA alloimmunization. Next, we planned to explore why only certain transfused patients become HLA alloimmunized by studying the association of various variables with HLA alloimmunization status. Such knowledge is critical to advance our understanding of the immunological processes underlying transfusion-related alloimmunization. This understanding could help lead to future prevention and treatment efforts to overcome the problem of alloimmunization.

BACKGROUND

Blood component transfusions are critical to the care of patients with SCD, severe aplastic anemia, thalassemia, and other hematologic diseases. Alloimmunization to RBC antigens places these patients at risk for potentially fatal hemolytic transfusion reactions and can dangerously restrict their access to life-saving blood. RBC alloimmunization, however, is not the only clinically serious alloimmunization problem. Most of these hematologic diseases can only be cured by a hematopoietic stem cell transplant (HSCT), a medical procedure that replaces a patient's blood-forming cells with a healthy donor's stem cells. If these patients undergo HSCT, alloimmunization to histocompatibility antigens from transfusions may complicate transplantation. In particular, alloimmunization to donor stem cell histocompatibility antigens can cause the transplant to fail through a process called graft rejection.

Most work regarding histocompatibility antigens involves the major histocompatibility complexes (MHC), which in humans are called human leukocyte antigens (HLA). HLA are cell surface proteins that are critical in determining transplant compatibility and mediating graft rejection. Minor histocompatibility antigens (mHA), however, also exist and are self-peptides presented by HLA. While not nearly as crucial as HLA for transplantation, mHA are the targets of the host-versus-graft response in HLA-matched transplants. Previous clinical work has already helped establish the notion that transfusion-related HLA alloimmunization can contribute to HLA-mismatched HSCT rejection (5-7, 14-21), but less is known about transfusion-related alloimmunization against minor histocompatibility antigens (mHA).

Experiments in animals have provided strong evidence of the capacity of transfusion-related mHA alloimmunization to induce HSCT rejection (8-13). First, using a MHC-matched canine HSCT model, Storb et al demonstrated that a single pre-HSCT whole blood transfusion from the donor significantly decreased the chance of successful engraftment. While 70% of dogs

not given a transfusion survived transplantation, only 8% of transfused dogs survived with most suffering from rejection (8). Storb et al also demonstrated that pre-HSCT blood transfusions from random unrelated donor dogs were similarly implicated in mediating MCH-matched HSCT rejection (9). Much of this early work, however, studied blood transfusions that did not undergo processing to remove leukocytes. Current transfusion practices in the United States now almost exclusively use RBC products that have undergone such removal of leukocytes to decrease the risk of febrile transfusion reactions and alloimmunization. Thus, it is important to note that Desmarets et al more recently demonstrated in a murine MCH-matched HSCT model that even RBC transfusions that undergo modern leukocyte reduction induce subsequent HSCT rejection (10). While 100% of mice given leukoreduced (LR) RBC transfusions from mHA-matched donors engrafted, only 6% of mice who received LR-RBC transfusions from mHA-mismatched donors engrafted. Leukocyte reduction of RBC transfusions thus does not appear to prevent mHA-directed HSCT rejection.

Clinical studies in patients with aplastic anemia have similarly suggested that pre-transplant transfusions may increase the risk of HLA-identical HSCT rejection (22-25). Using data from the Center for International Blood and Marrow Transplant Research, Champlin et al demonstrated that increasing numbers of pre-transplant transfusions were associated with an increased risk of graft failure among 262 patients who received conditioning with cyclophosphamide alone. Patients who received ≥40 transfusions had a relative risk of 2.9 (95% CI,1.5-5.8) of rejection compared to patients who had received <40 transfusions (24). While other studies have reported similar findings (22, 23, 25), this association does not establish that pre-transplant transfusions directly contribute to HSCT rejection. The amount of pre-HSCT transfusion support needed for these patients could simply have been an indicator of disease severity rather than a direct causative factor of rejection. Patients who received more transfusions may have a more altered immune system or damaged marrow environment that is not receptive to

transplantation. Alternatively, iron overload, rather than alloimmunization, from the transfusions may promote rejection. Also the above studies were performed in patients with aplastic anemia who all likely received both platelet and RBC transfusions, a similar association may not exist for patients with diseases like SCD who receive only RBC transfusions. Finally most of these studies were also done in the era before routine blood production leukoreduction and LR transfusions used today are likely not as potent a stimulus for mHA alloimmunization. It thus remains unclear whether in current clinical practice transfusions actually cause significant alloimmune responses to mHA.

Clinical investigation into mHA alloimmunization has been hampered by the lack of an assay suitable for assessing mHA alloimmunization. With the recent development of a multiplex antibody assay, it has become feasible to clinically study immunity to one group of mHA, H-Y antigens (26). H-Y antigens are encoded by the Y chromosome and thus expressed uniquely in males. They can elicit both cellular and humoral immune responses (27, 28) and have been implicated in the observed increased HLA-identical HSCT rejection rates of female patients who received male grafts (29-31). We have previously demonstrated an association of H-Y antibodies with secondary recurrent miscarriage (32), chronic graft-versus-host disease (33), and, most relevant to this study, renal transplant rejection (34). In an study of female renal transplant patients who received kidney transplants from male donors and later underwent renal biopsy for graft dysfunction, 79% of these patients who had had biopsy-proven acute rejection of their male grafts had de novo H-Y antibodies compared to only 8% of such patients with no evidence of rejection.

In this study we sought to determine if H-Y alloimmunization occurs after RBC transfusions. Though RBCs do not express H-Y antigens (35-37) (or HLA), RBC units contain other potentially immunogenic substances in addition to RBCs, most notably residual leukocytes that remain despite leukoreduction. These leukocytes would be expected to express both HLA

and mHA, including H-Y antigens if from a male blood donor. Since we would expect all heavily transfused patients to have been exposed to blood from male donors, we hypothesized that transfused females would develop H-Y antibodies while never transfused females would not have any H-Y antibodies. Transfused males were hypothesized to not have H-Y antibodies given that all males are expected to be tolerant to H-Y antigens.

Given the potential clinical importance of such immunization in this patient population, we conducted this study of H-Y alloimmunization in children with SCD. SCD is an inherited disorder of the hemoglobin protein in red blood cells that causes serious acute and chronic complications affecting multiple organs. It affects approximately 100,000 Americans and millions of individuals world-wide. Many people with SCD die before age 40 (3, 38-40), and in addition to this greater than 20 year reduction in life expectancy, many adults with SCD experience pain most days (1), have high rates of disability (41), and report poor overall quality of life (2, 42). Patients with SCD frequently require RBC transfusions to manage various SCD complications including cerebrovascular disease, aplastic crisis, and acute chest syndrome. HSCT is the only cure for SCD and has excellent engraftment results with the use of myeloablative conditioning and a HLA-identical sibling donor (43, 44). This approach, however, is not ideal: myeloablative conditioning has serious toxicities and the vast majority (>80%) of patients with SCD do not have a suitable HLA-matched sibling (43, 45, 46). HSCT using reduced-intensity conditioning and alternative donors are potential solutions to these problems, but both increase the risk of graft rejection. Initial HSCT studies for SCD with nonmyeloablative conditioning failed because of graft rejection (47, 48). More recently, HSCT studies for SCD using unrelated umbilical cord (49) and haploidentical bone marrow (50) donors have also reported very high (~50%) rates of graft rejection. Better understanding of the factors contributing to graft rejection including transfusion-related alloimmunization is critical to improving HSCT for SCD.

A secondary objective of our study was to more fully assess transfusion-related HLA alloimmunization in SCD patients, a matter of great relevance to current efforts to extend haploidentical HSCT to these patients (50). While it has been demonstrated that HLA alloimmunization is prevalent among multiply transfused patients with SCD (51-53), the contribution of transfusion therapy itself to HLA alloimmunization has not been well defined in this population in the era of RBC product leukocyte reduction. This is important since HLA alloimmunization can occur in the absence of known allo-exposure (i.e. transfusion, pregnancy or transplantation) (54, 55). Testing for HLA alloimmunization in a non-transfused group of SCD patients allowed us to attempt to gauge the contribution of transfusions to alloimmunity. For the transfused patients we also collected detailed information regarding their transfusion history in order to comprehensively analyze risk factors associated with transfusion-related HLA alloimmunization. Finally, we also performed immunophenotyping, using flow cytometry to measure the peripheral blood counts for a broad array of lymphocyte subsets, in attempt to identify novel immunological correlates. This study was thus designed to provide a rigorous assessment of HLA alloimmunization secondary to leukoreduced RBC transfusions, as well as provide the first clinical assessment of transfusion-mediated mHA alloimmunization.

METHODS

Research goal: Primary study aim was to determine if the proportion of female transfused patients with any H-Y antibodies was greater than the proportion of male transfused patients and female never transfused patients with any H-Y antibodies. Secondary study aims included: determining if a history of any RBC transfusion was associated with HLA antibody development; and, among transfused patients, what factors were associated with an increased risk of HLA antibody development.

Study Design, Participants and Sample Acquisition: We conducted a cross-sectional study of patients age ≥2 years with SCD SS or $S\beta^0$ at Children's Healthcare of Atlanta (CHOA) between October 2012 and May 2013. Approval was obtained by the by the Emory University and CHOA Institutional Review Boards. Three groups of patients were recruited:

1. Female patients on chronic RBC transfusion therapy (study group hypothesized to be at risk for alloimmunization to H-Y antigens from RBC transfusion)

2. Male patients on chronic RBC transfusion therapy (reference group hypothesized to be tolerant to H-Y antigens)

3. Female patients who had never received a transfusion (reference group thought to be not at-risk for alloimmunization to H-Y antigens).

Chronic transfusion patients had to have received greater than three consecutive RBC transfusions and had samples collected just prior to their scheduled transfusion. Patients in the never transfused group were roughly age-matched with chronic transfusion patients and specifically recruited at a routine clinic visit after a thorough record review showed that they had never received a transfusion. Pregnant patients or patients with a history of any pregnancy were excluded. To further avoid potential confounding sensitizing events, patients could not have

received a platelet transfusion or transplant. Patients were also excluded if they had a recent acute illness (defined as fever, emergency department visit, or hospitalization in the previous two weeks) or were taking an immunosuppressant medication other than hydroxyurea.

Measurements

Clinical Information: Information on history of splenectomy (yes/no) and hydroxyurea use (yes/no) were obtained from patient/family interview at time of consent and verfied through a review of medical records.

Blood Bank Data: Blood bank records were reviewed to obtain the total number of RBC units received (continuous variable) and history of RBC auto- or allo-antibodies (yes/no). All patients transfused during this time at our institution received exclusively packed RBC units that were pre-storagefilter leukocyte-reduced by the donor center. These units were non-irradiated, packed RBC units that were phenotypically matched for at least D, C/c, E/e, and K antigens (patients whose RBCs lacked the antigen were transfused with antigen-negative donor units). For patients who reported a history of transfusion at another institution, the patients' transfusion history from these centers was obtained directly from the outside institution blood bank in almost all cases. If an outside center could not be contacted then the transfusion history was obtained directly from the patient's family.

H-Y Antibody Testing (Primary Outcome): Serum was tested for immunoglobulin G (IgG) antibodies against 5 H-Y antigens (DDX3Y [DBY], EIF1AY, RPS4Y1, UTY, ZFY) using a previously validated H-Y protein microarray.(26) Slides were incubated with patient serum, washed, and then treated with a fluorescent secondary antibody which allowed the measurement of a mean fluorescence intensity (MFI) (continuous variable). Each H-Y-seropositive threshold (antibody present yes/no) was determined by MFI > the median MFI + 2.5 quartiles measured in 60 adult healthy males.

HLA Antibody Testing (Secondary Outcome): Serum was evaluated for anti-HLA IgG antibodies using the FlowPRA® screening test (One Lambda, Inc., Canoga Park, CA) as described previously (51). The panel reactive antibody (PRA) result from this test provides an estimate of the proportion of the general population to which the patient is alloimmunized to class I or class II HLA. Since the assay contains 30 class I and class II beads, a positive result is when 1/30 or >3% of the beads show channel shift emission. This outcome can thus be analyzed as either a numerical variable (0-100) or as a dichotomous variable (negative= <3%, positive= ≥3%). Separate results are given for HLA class I and HLA class II. To ensure accurate results, patients found to have a low positive PRA in which the fluorescent channel shift pattern was not distinct had the FlowPRA® screen repeated. These patients were also tested with LabScreen® Single Antigen Antibody Detection (One Lambda, Inc., Canoga Park, CA) for further result verification and identification of specific HLA antibodies. All HLA antibody testing was performed in the Emory University certified clinical HLA laboratory.

Immunophenotype Analysis: Eighteen different immune cell counts (continuous variable) were quantified on most patients in this study, as previously described in a separate publication (56). Immune phenotype analysis was performed on peripheral blood drawn into a Cytochex BCT tube (Streck Inc, Omaha NE). Quantitative evaluation of total white blood cell (WBC), lymphocytes, monocytes and polymorphonuclear leukocytes (neutrophils) was performed based on their CD45 expression versus side scatter (SSC) and compared to quantitative TruCount beads (BD Biosciences, San Jose CA). Lymphocytes were defined as $CD45^{high}/SSC^{low}$, monocytes as $CD45^{mid}/SSC^{mid}$, and neutrophils as $CD45^{low}/SSC^{high}$. The following lymphocyte populations were then identified immunologically: total CD3+ T cells (CD3+/CD20- lymphocytes), total CD20+ B cells (CD3-/CD20+ lymphocytes), NK cells (CD3-/CD20-/CD16+/$CD56^{high\&low}$), CD4+ T cells (CD4+/CD8- T cells), CD8+ T cells (CD8+/CD4- T cells) CD4+ putative T-regulatory cells (CD4+/ $CD25^{high}/CD127^{low}$). In addition, naïve T cells (Tn) were characterized

as CD45RA+/CCR7+; central memory (Tcm) as CD45RA-/CCR7+; effector memory (Tem) as CD45RA-/CCR7-; terminally-differentiated effector cells (Temra) as CD45RA+/CCR7- for both the CD4+ and CD8+ T cell populations. For comparative analysis, Tcm, Tem and Temra were combined and depicted as "T-memory". Naïve B cells were identified as CD27-/IgD+ and memory B cells as CD27+ CD38- IgD-. In addition, proliferation was measured by Ki-67 expression. Flow cytometry data was analyzed using FloJo software (TreeStar, Ashland OR).

Sample size and power considerations: This study was designed to detect a significant difference in the prevalence of transfused females with any H-Y antibodies compared to the prevalence in the two reference groups (transfused males and never transfused females). Based on our previous finding that 34% of transfused pediatric SCD patients had HLA antibodies (51), we assumed a similar prevalence of H-Y alloimmunization in transfused females and a low prevalence (5%) in each of the two reference groups. To demonstrate this proposed 29% absolute prevalence difference with 80% power and 95% confidence, a sample size of 50 transfused females and 25 patients in each of the reference groups was required using Fisher's exact method.

Analytic Plan

Descriptive analyses: Important demographic (mean age) and clinical information (mean number of RBC transfusions, % with history splenectomy, % with hydroxyurea use) were determined for each of the three study groups to assess baseline differences in the groups.

Primary Analysis: To compare the prevalence of any H-Y antibodies in each of the three patient groups, a Fisher's exact test was used.

Secondary Analysis I: To evaluate the effect of RBC transfusion on HLA antibody development, a chi-square test or Fisher's exact test when appropriate was used to compare the prevalence of HLA class I or HLA class II antibodies in transfused versus non-transfused patients. This comparison was done classifying HLA class I or II alloimmunization as present if any HLA

antibody was detect on the screen (PRA≥3%) and also for only high positive antibody screens (PRA>25%).

Given that a difference in the proportion of HLA antibodies in the transfused versus non-transfused groups could be due to possible confounding variables of age, history of splenectomy, or hydroxyurea use, a univariate analysis was next done comparing patients who had HLA class I or HLA class II antibodies with those patients who did not have such antibodies. Comparisons were made via the chi-square test or Fisher's exact test when appropriate for categorical variables and two-sample *t*-test for continuous variable (age was normally distributed). Multivariable analysis was then performed via logistic regression to determine if the effect of RBC transfusion history remained significant when controlling for three other variables that may affect immune function and thus antibody formation: age, history of splenectomy, and use of hydroxyurea

Secondary Analysis II: To evaluate risk factors for strong HLA class I alloimmunization among the transfused patients, patients were categorized as being strongly HLA alloimmunized (HLA class I PRA >25%) or not (HLA class I PRA ≤25%). A univariate analysis was done comparing a number of transfusion-related variables among patients with HLA class I PRA >25% compared to patients with HLA class I PRA ≤25%. Comparisons were made via the Fisher's exact test or chi-square test for categorical variables and two-sample *t*-test or Wilcoxon rank-sum test when appropriate for continuous variables. A multivariable analysis was then performed using the variables deemed clinically most important to determine if any variables remained significant when controlling for the other variables:

Statistical calculations were performed with SAS 9.3 (SAS Institute Inc., NC.) and graphics created using GraphPad Prism version 6.02 (GraphPad Software, La Jolla California USA, www.graphpad.com).

RESULTS

Patient characteristics: A total of 117 patients with SCD were enrolled. Three patients were excluded because they did not meet eligibility criteria upon further review of the medical record, yielding 58 females on chronic transfusion, 32 males on chronic transfusion, and 24 never transfused females (Table 1). Patients' age ranged from 2.1 to 22.1 years and was similar across groups (mean age in years for transfused female 12.0, standard deviation 4.4; transfused male 12.0, standard deviation 4.3; never transfused female 10.5, standard deviation 4.6; p=0.37). More patients in the chronic transfusion groups had a history of splenectomy (27% vs 0%, p=0.004), while more patients in the never transfused group were taking hydroxyurea (46% vs 8%, p<0.0001).

Among the chronic transfusion patients, the number of RBC units transfused varied considerably with a median of 93 units received (min 6, max 543 units). The main indications for chronic transfusion therapy were primary (59%) and secondary stroke prevention (32%). Regarding RBC antibody history, 29/90 (32%) had a history of a RBC alloantibody, 25/90 (28%) had a history of a RBC autoantibody, and 19/90 (21%) had a history of both. The majority of patients, 56/90 (62%), were exclusively transfused at our institution. Of the patients who had received a transfusion outside of our institution, only two patients had received a non-leukoreduced RBC transfusion (neither of these patients had H-Y or HLA antibodies).

H-Y antibodies were not associated with RBC transfusion: The prevalence of patients with any H-Y antibodies was low in each of the three study groups: 4/58 (7%) of transfused females, 2/32 (6%) of transfused males, and 1/24 (4%) of never transfused females (p=1.0, Table 2). Mean MFI was also not significantly different between the three study groups for any of the H-Y antigens (Figure 1). The few patients classified as antibody positive had MFI values just above the positive threshold and much lower than the MFI values from positive control reference

samples obtained from male HSCT patients who had received a female graft (F → M) who were known to have H-Y alloantibodies (Figure 2).

HLA class I antibodies were associated with RBC transfusion: Figure 3 shows the distribution of HLA class I and II PRA values for the transfused and never transfused patients. Regarding HLA class I antibody prevalence, 30/90 (33%) of transfused and 3/24 (13%) of never transfused patients had HLA class I antibodies (p=0.046). Considering only patients with a high class I PRA, 16/90 (18%) of transfused and none of the never transfused patients had a HLA class I PRA value >25% (p=0.022). HLA class II antibodies were less common and not different between the two groups: 6/90 (7%) of transfused and 2/24 (8%) of never transfused patients had HLA class II antibodies (p=0.67). Only three patients (all transfused patients) had both HLA class I and class II antibodies.

Table 3A shows the univariate analysis of HLA class I alloimmunization among all patients. Both of the transfused study groups had a higher odds ratio (OR) of having HLA class I antibodies compared to the reference female never transfused patient group, although this difference was only significant for the male chronic transfusion study group. When the two transfused groups were combined in that only the dichotomous transfusion variable (history of any RBC transfusion, yes/no) was evaluated, transfusion was significantly associated with HLA class I alloimmunization after adjustment for age, splenectomy, and hydroxyurea (Table 3B). In contrast, no variable was significantly associated with HLA class II alloimmunization (Table 4A-B).

HLA antibodies in never transfused patients: To validate the finding that 5/24 (21%) of never transfused, nulliparous patients had a positive HLA antibody screen (class I or class II), these these positive results were confirmed by two methods. First, re-testing banked serum from the original collection with a repeat antibody screen yielded identical positive PRA results. Second, a

different HLA antibody assay involving single antigen bead testing was also performed on these samples and identified antibodies to unique HLA antigens for each patient (Table 5). In addition, a second serum sample was collected from these patients 6.0-18.1 months after the first sample for another HLA antibody screen. On this repeat testing, 3 patients had similar positive results and 2 patients had a distinct change in the flow emission pattern and a decreased PRA consistent with diminishing antibody (Figure 4).

In chronic transfusion patients, history of a RBC alloantibody but not transfusion burden was associated with HLA class I alloimmunization: Among the chronic transfusion patients, alloimmunization to HLA was not associated with number of lifetime transfusion exposures. While patients who had received the most number of transfusions had a higher prevalence of RBC alloimmunization, a similar trend did not exist for HLA alloimmunization (Table 6). Of note, two patients who had received less than 20 RBC units had among the highest HLA class I PRA values (91% and 97%), while 12/16 (75%) of the patients who had received the most transfusions (>200 RBC units) did not have any HLA antibodies.

To further investigate why some transfused patients became alloimmunized to class I HLA, a HLA class I PRA >25% was used to define a group of patients who were strongly HLA alloimmunized. Chronic transfusion patients with a HLA class I PRA >25% (n=16) were thus compared with chronic transfusion patients who had a HLA class I PRA ≤ 25% (n=74). In both the univariate and multivariable analyses, the only variable significantly associated with HLA class I alloimmunization was a history of a RBC alloantibody (Table 7A-B).

Immunophenotype analysis of chronic transfusion patients: We have previously reported on the immune phenotype of SCD patients that included the patients we describe in this study (56). In the current analysis, we compared the 18 studied immune cell counts between chronic transfusion patients with a HLA class I PRA >25% (n=13) vs chronic transfusion patients with

HLA class I PRA ≤25% (n=59) who had samples collected for this testing and were not on concurrent hydroxyurea therapy (Table 8). The few patients on concurrent hydroxyurea therapy were excluded in this analysis since we have previously shown that hydroxyurea treatment appears to significantly affect most immune cell counts (56). This analysis revealed that the number of memory B cells was significantly different between the groups, with the highly sensitized patients having more memory B cells (44 vs 29 cells/ul, p=0.019) .

Longitudinal analysis of HLA Antibodies in chronic transfusion patients: Sixteen chronic transfusion patients in this study had data on HLA alloimmunization from our previous study (51). At the time of this previous study, 14 patients were negative for HLA antibodies and 2 patients were positive for HLA antibodies. Despite a median of 146 additional RBC units transfused over a median of 5.5 years, 11/14 (79%) of patients with initially negative HLA antibody screens continued to have no HLA antibodies detected. After a median of 78 additional RBC units transfused over a median of 5.1 years, 3/14 (21%) of patients developed new HLA antibodies. The 3 patients who developed interval positive HLA antibody screens had the following positive PRA results: patient #1 class I 8%, patient #2 class I 17%, patient #3 class II 41%. Both of the 2 patients who initially had positive HLA antibody screens (both with class I PRA >25%) continued to have a class I PRA value >25% on repeat testing 5.6 and 7.0 years later.

DISCUSSION

Our results suggest that leukocyte-reduced RBC transfusions promote HLA antibody formation, but they do not cause detectable H-Y alloantibodies. Thus, while leukoreduced RBC transfusions can lead to HLA alloimmunization, they may be an inadequate stimulus for H-Y alloimmunization. Supporting this idea, Desmerets et al found in a murine model that leukocyte reduction of pre-transplant RBC transfusions nearly obviated subsequent H-Y directed HSCT rejection (10). An important caveat to this interpretation is that in the current study only humoral immunity was assessed. Thus, it is possible that leukoreduced RBC transfusions could induce an H-Y response involving only T cells as appears to be characteristic of immunity to other mHAs (12, 57). Moreover, it should not be inferred from the lack of an association between RBC transfusions and H-Y antibody formation in our study that leukoreduced RBC transfusions are weakly or non-immunogenic for all mHAs. Previous murine work suggests that pre-transplant leukocyte-reduced RBC transfusions mismatched for multiple autosomal mHAs can strongly induce HSCT rejection (10), and immunity to mHAs other than H-Y antigens was not evaluated in the current study.

This study builds on our previous description of HLA alloimmunization in SCD (51) by more definitively linking RBC transfusion to the production of HLA class I antibodies. While pregnancy can definitively cause HLA alloimmunization, no patient in this study had a known pregnancy or spontaneous abortion. It is very unlikely that undocumented pregnancy-induced alloimmunization affected our results as most females studied were premenarchal, older patient age was not associated with alloimmunization, and males actually had a higher prevalence of HLA antibodies. Given that patients on chronic transfusion therapy are clinically different (have more severe disease) than patients who have never received a RBC transfusion, it is possible that other variables different between the two groups other than RBC transfusion could affect the formation of HLA antibodies. When two important variables that affect aspects of the immune

system (hydroxyurea use and history of splenectomy) were considered, however, they did not alter the significant association between RBC transfusion history and HLA class I alloimmunization.

Our findings thus add to the growing body of evidence that HLA alloimmunization after RBC transfusion remains a problem despite leukocyte reduction (7, 58). The source of the HLA class I antigens in RBC units causing sensitization is currently unknown. However, even with modern leukocyte reduction as many as 5×10^6 leukocytes may remain in a RBC unit, and these residual leukocytes may lead to sensitization. Residual platelets (which express class I HLA) and free-floating HLA molecules also contaminate all RBC units. Furthermore, RBCs themselves may also contain adsorbed HLA antigens on their surface (59, 60). Future work should seek to characterize the HLA-sensitizing source in RBC units, with a goal of decreasing this cell subset from processed leukoreduced blood products.

Our finding that some non-transfused SCD patients had HLA antibodies is consistent with others' reports of "natural" or "spontaneous" HLA alloimmunization in "unsensitized" individuals (54, 55). The etiology of these antibodies is unknown; their production may be triggered by a non-allogeneic stimulus such as immunization (61-63) or cross-reactivity to a foreign antigen such as a microorganism (64-66). While also found in healthy individuals (54), it is unclear if these antibodies are more common in patients with SCD. Our limited data suggests that these apparently unprovoked antibodies may be more transient than those elicited by RBC transfusion, as the reactivity of two of these five patients waned over months, compared to the stability of HLA antibodies observed in two transfused patients for more than five years.

In this study, as in our previous work (51), the lone factor associated with HLA alloimmunization among multiply transfused patients was a history of a RBC alloantibody. Our results add further to the evidence linking HLA and RBC alloimmunization.[31,40,53] Recent studies

suggest that genetic and immunologic differences distinguish these immunologic "responders" (patients who make alloantibodies) from "non-responders" in SCD (67-69). Our observation that strongly HLA alloimmunized patients had higher memory B cell counts suggests that an underlying difference in the B cell biology of these patients may explain their tendency to form or not form alloantibodies. However, it is also possible that this association was found by chance as we did not account for multiple comparisons in our analysis. It is also possible that the higher memory B cell counts are a result rather than a cause of the HLA immunity. In addition, while SCD transplant studies to date have not found a clear association between the number of transfusions received and graft rejection (70), it is possible that an individual's unique response to transfusion (more so than the total number of transfused units) may impact graft rejection.

One consideration in interpreting our data is that in a post hoc fashion we used an HLA class I PRA >25% to define an allo-antibody "responder." We chose this threshold after observing that none of the never transfused patients had a HLA class I PRA value above this level. A high PRA value has clinical significance in that it suggests that a patient has HLA antibodies that react with a large percentage of the general population. However, a high PRA does not necessarily mean that a patient has many unique HLA antibodies or has antibodies at a high titer.

Given the relatively high prevalence of patients with HLA class I or class II antibodies in our transfused group (37%) and the known association of donor-specific HLA antibodies with graft rejection (14-21), our results provide strong rationale for performing HLA antibody testing in patients with SCD being evaluated for HSCT using HLA-mismatched related or unrelated donors. With the promising early results (50, 71) of haploidentical transplant for SCD and potential of this approach to cure many patients with SCD, the role of HLA alloimmunization could become increasingly important in SCD as HLA antibody testing should be used to help select donors (so as to avoid donors that have HLA mismatches that would react against a patient's specific HLA antibodies). Strategies also need to be developed to counter

alloimmunization for those patients whose only option is a donor they are sensitized against. One area that should be explored further is desensitization treatment, as various protocols (including plasmapheresis and intravenous immune immunoglobulin) (72) have been implemented in which successful HSCT was achieved despite donor specific antibodies (73).

Furthermore, all SCD patients undergoing HSCT may benefit from HLA antibody testing as HLA alloantibodies predispose transplant patients to platelet refractoriness (74, 75). Pre-transplant HLA antibodies can persist for months following reduced intensity HSCT leading to prolonged platelet transfusion refractoriness (76). Since SCD patients are at increased risk of cerebral hemorrhage during HSCT (77), thrombocytopenia is a major concern and platelet transfusions are thus given to patients with SCD much more often than other HSCT patients in the post-transplant period (typically to maintain a platelet count $>50 \times 10^9$ / L). If a patient has alloantibodies against certain class I HLA then obtaining platelet units that do not express those HLA would likely make it easier to maintain an adequate platelet count to prevent hemorrhagic complications in this at-risk patient population. This practice would also likely minimize the number of platelet transfusions which could offer additional clinical benefits by avoiding potential deleterious effects of transfusion (allergic and febrile reactions, volume overload, transfusion-related acute lung injury).

In conclusion, this study demonstrated that in pediatric SCD leukocyte-reduced RBC transfusions are associated with HLA class I alloimmunization but not H-Y antibody development. Importantly, some individuals ("responders") had a high risk of becoming immunized to both RBC and HLA antigens, while others ("non-responders") never become immunized despite large transfusion burdens. Future studies investigating responder versus non-responder patients with SCD in the context of transfusion and HSCT outcomes are needed. These studies will require close collaboration between hematology, transplant, and transfusion services, with a common goal of improving outcomes for patients with SCD.

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TABLES / FIGURES

Table 1: Demographic and Clinical Characteristics by Study Group

	Chronic Transfusion Female n= 58	Chronic Transfusion Male n= 32	Never Transfused Female n= 24
Age, mean (min, max)	12.0 (2.1, 19.4)	12.0 (5.0, 22.1)	10.5 (2.5, 18.4)
RBC units transfused, mean (min, max)	116 (6, 543)	129 (13, 531)	0 (0, 0)
Splenectomy	11 (19.0%)	13 (40.6%)	0 (0%)
Hydroxyurea	5 (8.6%)	2 (6.3%)	11 (45.8%)

Table 2: Prevalence of H-Y Antibodies by Study Group

H-Y antigen	Chronic Transfusion Female n= 58	Chronic Transfusion Male n= 32	Never Transfused Female n= 24
DBY	1 (1.7%)	0 (0%)	0 (0%)
EIF1AY	0 (0%)	0 (0%)	0 (0%)
RPS4Y1	2 (3.4%)	0 (0%)	0 (0%)
UTY	2 (3.4%)	2 (6.3%)	1 (4.2%)
ZFY	1 (1.7%)	0 (0%)	0 (0%)
Any H-Y	4 (6.9%)	2 (6.3%)	1 (4.2%)

Table 3A: Univariate Analysis of HLA class I alloimmunization

	HLA class I positive n=33	HLA class I negative n=81	OR (95% CI)	P-value
Study group:				0.037
Female chronic transfusion	16 (27.6%)	42 (72.4%)	2.7 (0.70-10.2)	
Male chronic transfusion	14 (43.8%)	18 (56.3%)	5.4 (1.3-22.0)	
Female never transfused	3 (12.5%)	21 (87.5%)	1.0 (reference)	
RBC transfusion	30 (90.9%)	60 (74.1%)	3.5 (0.97-12.7)	0.046
Age, mean (min, max)	12.1 (2.1, 20.7)	11.5 (2.1, 22.1)	1.03 (0.94-1.1)	0.49
Splenectomy	5 (15.2%)	19 (23.5%)	0.58 (0.20-1.7)	0.32
Hydroxyurea	4 (12.1%)	14 (17.3%)	0.66 (0.2-2.2)	0.49

P-value calculated from Fisher's exact test or chi-square test for categorical variables and two-sample *t*-test for continuous variable.

Table 3B: Multivariable analysis of HLA class I alloimmunization

	OR (95% CI)	p-value
RBC transfusion (ref: no transfusion)	4.4 (1.1-18.0)	0.042
Age (ref: per year of age)	1.03 (0.93-1.1)	0.61
Splenectomy (ref: no splenectomy)	0.42 (0.14-1.3)	0.13
Hydroxyurea (ref: no Hydroxyurea)	1.1 (0.29-4.4)	0.86

P-value calculated from Wald chi square test.

Table 4A: Univariate Analysis of HLA class II alloimmunization

	HLA class II positive n=8	HLA class II negative n=106	OR (95% CI)	P-value
Female chronic transfusion	5 (8.6%)	53 (91.4%)	1.04 (0.19-5.8)	0.63
Male chronic transfusion	1 (3.1%)	31 (96.9%)	0.36 (0.030-4.2)	
Female never transfused	2 (8.3%)	22 (91.7%)	1.0 (reference)	
RBC transfusion	6 (75.0%)	84 (79.2%)	0.79 (0.15-4.2)	0.67
Age, mean (min, max)	10.5 (2.1, 15.6)	11.8 (2.1, 22.1)	0.94 (0.80-1.1)	0.45
Splenectomy	0 (0%)	24 (22.6%)	-	0.20
Hydroxyurea	1 (12.5%)	17 (16.0%)	0.75 (0.086-6.5)	1.0

P-value calculated from Fisher's exact test or chi-square test for categorical variables and two-sample *t*-test for continuous variable. Unable to calculate OR of class II HLA alloimmunization for splenectomy because all 8 patients with class II antibodies did not have a splenectomy

Table 4B: Multivariable analysis of HLA class II alloimmunization

	OR (95% CI)	p-value
RBC transfusion (ref: no transfusion)	0.96 (0.15-6.2)	0.97
Age (ref: per year of age)	0.95 (0.80-1.1)	0.51
Splenectomy (ref: no splenectomy)	-	0.95
Hydroxyurea (ref: no Hydroxyurea)	0.58 (0.052-6.6)	0.66

P-value calculated from Wald chi square test. Unable to calculate OR of class II HLA alloimmunization for splenectomy because all 8 patients with class II antibodies did not have a splenectomy.

Supplemental Table 5: Never Transfused Patients with HLA antibodies

Pt #	Age (yrs)	Antibodies identified*	MFI min, max	Initial PRA		Time interval (mo)	Second PRA		Significant Interval Change†
				Class I (%)	Class II (%)		Class I (%)	Class II (%)	
19	15	B51, B52, B72, B77	2283, 5643	19	0	11.1	8	0	Yes, decreased
21	5	DQ2	11848	0	37	10.6	0	35	No
22	6	A31, A30	3807, 16750	7	0	9.7	7	0	No
81	10	DR4, DR7, DR9	3745, 6195	0	16	18.1	0	9	Yes, decreased
86	14	A25, A26, A43, A66, B73	2662, 8102	6	0	6.0	5	0	No

*Antibodies identified via single antigen bead testing on the initial sample. †Significant interval change determination based on PRA flow emission pattern (see Figure 4).

Table 6: Prevalence of RBC and HLA Alloimmunization in Chronic Transfusion Patients by Transfusion Burden Quartiles

# of RBC units received	% with RBC alloantibodies	% with HLA alloantibodies (class I and II)	% with HLA class I alloantibodies	% with HLA class I PRA >25%
6-40	5/23 (21.7%)	7/23 (30.4%)	7/23 (30.4%)	4/23 (17.4%)
41-92	5/22 (22.7%)	9/22 (40.9%)	8/22 (36.4%)	2/22 (9.1%)
93-176	8/23 (34.8%)	10/23 (43.5%)	9/23 (39.1%)	5/23 (21.7%)
>177	11/22 (50.0%)	7/22 (31.8%)	6/22 (27.3%)	5/22 (22.7%)

Table 7A: Univariate Analysis of HLA class I PRA high positive among Chronic Transfusion Patients

	HLA class I PRA high positive n= 16	HLA class I PRA low positive or negative n= 74	OR (95% CI)	P-value
Female	9 (56.3%)	49 (66.2%)	0.66 (0.22-2.0)	0.45
Age, mean (min, max)	12.6 (4.6, 20.7)	11.8 (2.1, 22.1)	1.0 (0.92-1.2)	0.49
# of RBC units transfused, mean (min, max)	130 (9, 324)	119 (6, 543)	1.0 (0.996-1.006)	0.55
Age began chronic transfusion, mean (min, max)	6.5 (1.1, 10.8)	6.2 (0.5, 17.5)	1.0 (0.89-1.2)	0.38
Clinical stroke	8 (50.0%)	21 (28.4%)	2.5 (0.84-7.6)	0.093
Splenectomy	3 (18.8%)	21 (28.4%)	0.58 (0.15-2.3)	0.54
RBC alloantibody	11 (68.8%)	18 (24.3%)	6.8 (2.1-22.3)	0.0006
Outside institution transfusion	6 (37.5%)	28 (37.8%)	0.99 (0.32-3.0)	0.98
Transfusion during acute chest syndrome	4 (25.0%)	29 (39.2%)	0.52 (0.15-1.8)	0.29

PRA high positive defined as >25%. P-value calculated from Fisher's exact test or chi-square test for categorical variables and two-sample *t*-test or Wilcoxon rank-sum test for continuous variables.

Table 7B: Multivariable Analysis of HLA class I PRA high positive among Chronic Transfusion Patients

	OR (95% CI)	P-value
Sex (ref: male)	0.79 (0.22-2.8)	0.72
Age (ref: per year of age)	1.04 (0.72-1.5)	0.85
# of RBC units transfused (ref: per unit transfused)	0.998 (0.99-1.01)	0.75
Age began chronic transfusion (ref: year of age)	0.97 (0.72-1.3)	0.85
Clinical stroke (ref: no stroke)	1.7 (0.45-6.3)	0.44
Splenectomy (ref: no splenectomy)	0.52 (0.092-2.9)	0.46
RBC alloantibody (ref: no RBC alloantibody)	6.2 (1.8-22.0)	0.004

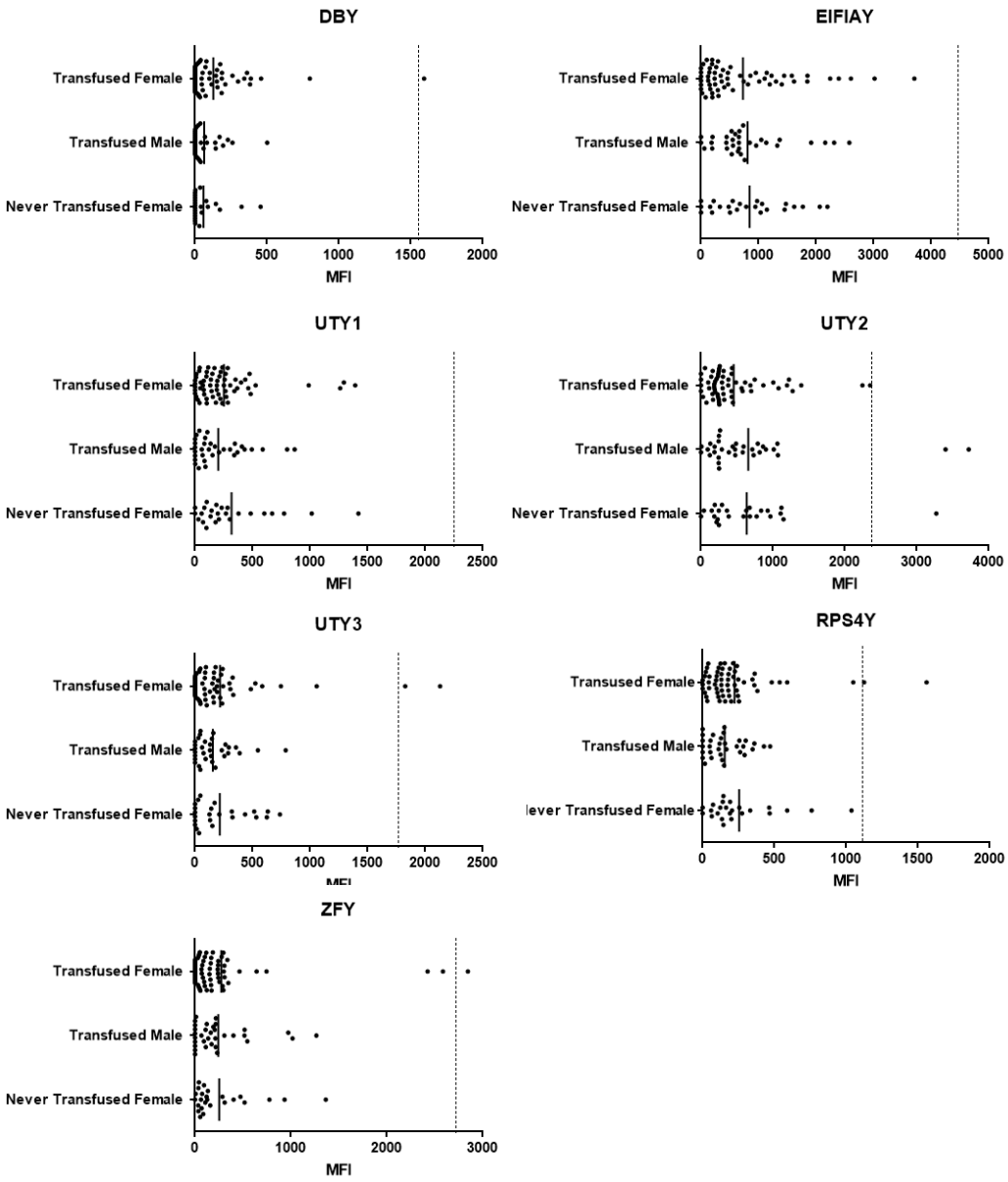
PRA high positive defined as >25%. P-value calculated from Wald chi square test.

Table 8: Univariate Analysis of Immune Cell Counts as Marker for HLA class I PRA high positive among Chronic Transfusion Patients

	HLA class I high positive n=13 mean (sd) cells/ul	HLA class I low positive or negative n=59 mean (sd) cells/ul	P-value
WBC	19186 (10589)	15366 (6221)	0.28
Neutrophils	13098 (9166)	9706 (4925)	0.34
Monocytes	1496 (778)	1233 (596)	0.27
Lymphocytes	4280 (1318)	4023 (1878)	0.44
B Cells	1120 (474)	1260 (1006)	0.94
T Cells	2582 (959)	2302 (921)	0.35
NK Cells	419 (243)	310 (170)	0.10
Naïve B	615 (343)	673 (600)	0.98
Memory B	44 (21)	29 (21)	0.019
CD4	1515 (546)	1399 (578)	0.51
CD8	757 (390)	651 (347)	0.34
CD4 T-naïve	686 (348)	703 (424)	0.91
CD4 T-memory	828 (330)	695 (263)	0.18
CD4 T-reg	117 (44)	109 (45)	0.52
CD8 T-naïve	353 (234)	335 (198)	0.85
CD8 T-memory	404 (302)	315 (224)	0.23
CD4 Ki67	51 (32)	42 (29)	0.37
CD8 Ki67	16 (12)	16 (18)	0.84

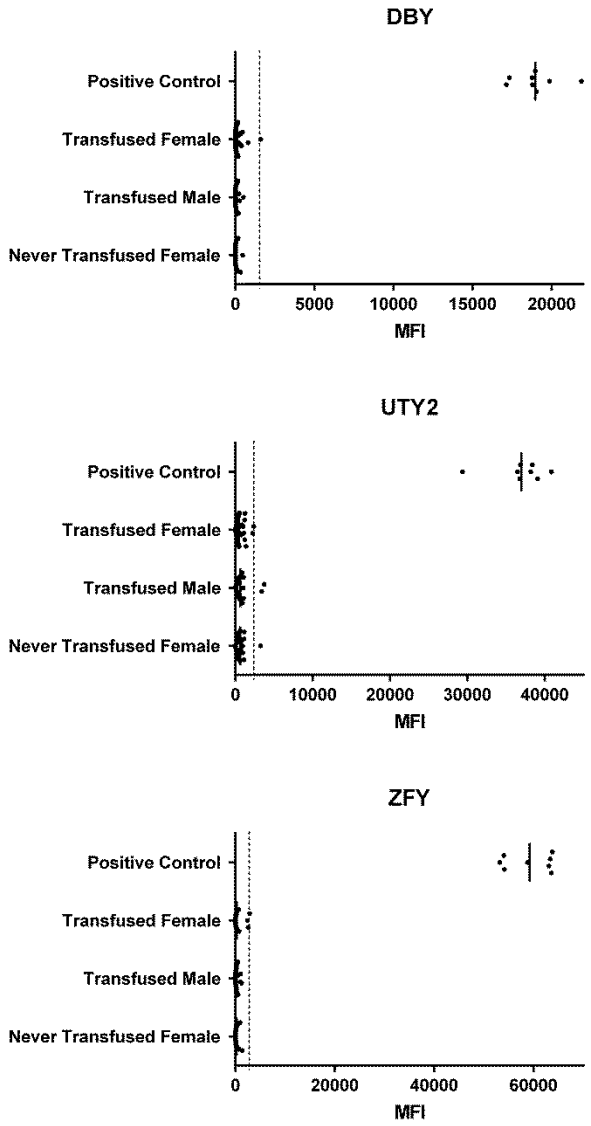
18 patients excluded from this analysis (11 for no flow sample, 6 for concurrent HU therapy, 1 for technical error). P-value calculated from two-sample *t*-test on log10 transformed cell count data.

Figure 1: Comparison of the MFI for antibodies against 5 H-Y antigens for the three study groups



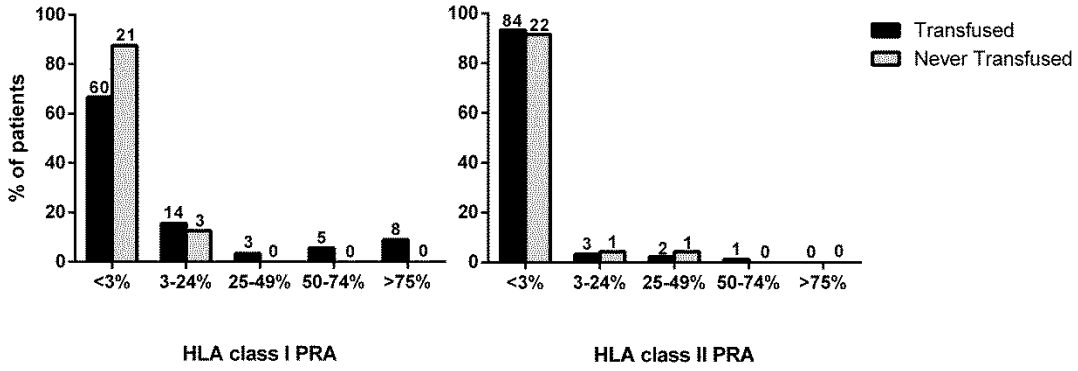
MFI= mean fluorescence intensity. UTY antigen was tested with three different proteins (UTY1, UTY2, UTY3) given the large size of the antigen. The solid lines for each group depict the group mean MFI value. The dashed line represents the positive MFI threshold that defined seropositivity calculated from median MFI + 2.5 quartiles measured in 60 adult healthy males.

Figure 2: Comparison of the H-Y Antibody MFI for the three study groups with positive controls



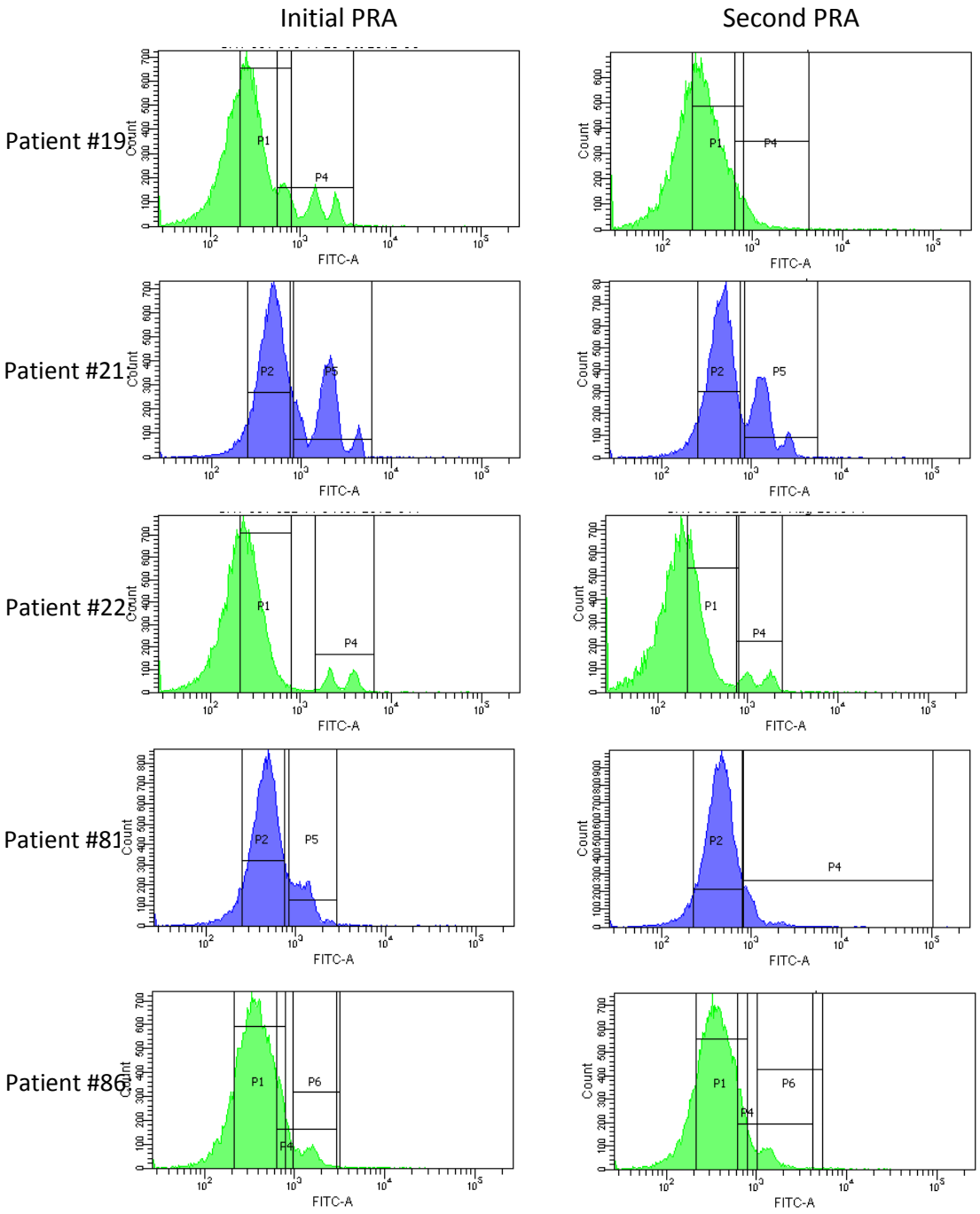
Banked plasma from male HSCT patients who received a female graft (F→M) who were known to have H-Y antibodies was used as a positive control. Results are only shown for DBY, ZFY, and UTY2 because these HSCT patients did not make antibody against EIFIAY, RPS4Y, UTY1, or UTY3. The solid lines for each group depict the group mean MFI value. The dashed line represents the positive MFI threshold that defined seropositivity calculated from median MFI + 2.5 quartiles measured in 60 adult healthy males.

Figure 3: HLA PRA Values in Transfused vs Never Transfused Patients



Distribution of PRA values in the 90 transfused (black) and 24 never transfused (gray) patients. Bar heights depict the percentage of patients in that group (transfused or never transfused) having the specific PRA range. Numbers above the bar denote the absolute number of patients within that PRA range.

Figure 4: Change in Panel Reactivity Antibody Flow Cytometry Plots for Never Transfused Patients Over Time



Initial and second PRA plot for the five never transfused patients with HLA antibodies. Class I PRA plot shown for patient #19, #22, and #86. Class II PRA plot shown for patient #21 and #81.