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HLA Alloimmunization in Pediatric Sickle Cell Disease and Thalassemia Major:

Prevalence and Risk factors

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Abstract

HLA Alloimmunization in Pediatric Sickle Cell Disease and Thalassemia Major: Prevalence and Risk factors

By Marianne Elaine McPherson, M.D.

Individuals who require blood transfusion are at risk for alloimmunization against foreign human antigens. Alloimmunization to red blood cells (RBC) occurs commonly in sickle cell disease (SCD) and thalassemia major and limits the ability to safely transfuse blood. Alloimmunization to human leukocyte antigens (HLA) can occur with RBC transfusions and may result in severe transfusion reactions and graft rejection in organ and stem cell transplantation. The prevalence and risk factors for HLA alloimmunization are unknown in SCD and thalassemia patients requiring frequent transfusions. A cross-sectional study of HLA alloantibodies in pediatric patients with SCD and thalassemia major was performed to test the hypothesis that HLA alloimmunization occurs at a frequency equal to or greater than RBC alloimmunization. All thalassemia major patients in Atlanta were tested. SCD patients were selected to match for presence or absence of RBC antibodies and chronic transfusion therapy. Over a 3-year period, 17 thalassemia major and 73 SCD patients were sampled. HLA alloimmunization was seen in 53% of SCD patients with RBC antibodies and 21% without RBC antibodies (OR 4.32 [1.55 – 12.1]). In multivariate logistic regression, RBC antibodies were predictive of HLA antibodies and showed interaction with chronic transfusion exposure. In thalassemia patients, the prevalence of HLA alloimmunization was 47%. This analysis is the first description of HLA alloimmunization in SCD patients and in pediatric thalassemia major patients transfused in the U.S. and demonstrates that individual predisposition to alloimmunization is common for both RBC and HLA antigens.

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Tables

INTRODUCTION

Patients with sickle cell disease (SCD) and thalassemia major are dependent on red blood cell (RBC) transfusions for treatment of life-threatening anemia and disease complications. A potential complication of multiple blood transfusions is the formation of antibodies directed against foreign blood antigens, an event termed alloimmunization. Alloimmunization to foreign RBC surface antigens has serious consequences in transfusion-dependent patients, potentially resulting in hemolytic transfusion reactions and limiting the ability to deliver compatible blood products when a patient urgently needs transfusion. In SCD, alloimmunization to RBC occurs much more frequently than in thalassemia or in transfused individuals without hemoglobinopathy (1-3). Although many factors are likely to contribute to alloimmunization, including genetic predisposition, inflammation, and exposure to phenotypically-dissimilar blood donors, it remains unclear why some individuals frequently form alloantibodies in response to blood transfusion while other individuals never elicit an antibody response to transfusion despite repeated exposure to foreign blood antigens.

Blood transfusion exposes a recipient to not only foreign RBC but also many "non-self" antigens. Human leukocyte antigens (HLA) are one of the most diverse groups of antigens in the human genome with considerable interpersonal variability, allowing the immune system to distinguish self versus non-self. Alloimmunization to HLA is a common occurrence following exposure to foreign tissue (e.g. pregnancy and organ transplantation). Because HLA molecules are not expressed by mature RBC, their role in RBC transfusion has been considered to be insignificant. However, HLA

alloimmunization due to RBC transfusion has been well-documented and may have adverse clinical consequences in patients with SCD and thalassemia major.

The clinical impact of HLA alloantibodies is well established among transplant patients, in whom HLA antibodies mediate hyper-acute graft rejection and delayed graft failure. SCD and thalassemia major patients with severe disease complications are candidates for hematopoietic stem cell transplantation (HSCT), and less commonly may be considered for cardiac or renal transplantation due to severe chronic organ damage. High-titer HLA alloantibodies are considered an absolute contraindication to organ transplantation and pose serious risks in myeloablative HSCT; therefore HLA alloimmunization from past RBC transfusions may prevent the sickest SCD and thalassemia major patients from benefiting from life-saving transplantation. A third scenario in which HLA alloantibodies may be detrimental is blood transfusion reactions. While most life-threatening hemolytic transfusion reactions are mediated by RBC alloantibodies, anecdotal evidence has led to speculation that HLA alloantibodies also may play a role in some cases of severe delayed hemolytic transfusion reactions.

In summary, HLA alloimmunization is detrimental to patients with life-long dependence on blood transfusion, HSCT, and organ transplantation, yet the prevalence of HLA alloantibodies among SCD and thalassemia patients is unknown. Since the causative factors for alloimmunization are not well understood, it is unknown if RBC and HLA alloimmunization have common risk factors. Just as the causative factors for RBC alloimmunization are unknown, likewise the risk factors for HLA alloimmunization are unknown. The primary aim of this cross-sectional analysis is to establish the prevalence of HLA alloantibodies in multiply-transfused patients with SCD or thalassemia major.

There are two additional aims for the analysis of SCD patients. The first is to determine the association of HLA alloimmunization with RBC antibodies among SCD patients; the second is to identify other clinical variables that may predispose individuals to HLA alloimmunization.

BACKGROUND

Sickle cell disease (SCD) and thalassemia major (Cooley's anemia) are inherited hemoglobin disorders that are defined by severe anemia, chronic organ damage and ultimately early mortality. Although SCD and thalassemia are uniquely different diseases in terms of clinical complications and organ damage, in both disorders the mainstay of treatment is RBC transfusion. Chronic use of RBC transfusions, although life-sustaining, has multiple complications, including iron overload and alloimmunization, and is not sufficient to completely eliminate disease complications. The only curative therapy for SCD and thalassemia is allogeneic hematopoietic stem cell transplantation (HSCT). Over 250 individuals with SCD and 1600 individuals with thalassemia major worldwide have received HSCT with high rates of successful engraftment and survival using related HLA-identical donors (4, 5). Because complications and mortality from HSCT are much greater when alternative stem cell donors (unrelated or partially HLA-matched donors) are used, the use of HSCT has been mainly restricted to individuals with HLA-identical sibling donors, however clinical trials currently are testing the use of alternative donors to allow more patients with SCD and thalassemia to benefit from HSCT (6).

Thalassemia is a common disorder worldwide, mainly affecting individuals of Middle Eastern, Mediterranean, Indian, and Southeast Asian descent. In its most severe form, thalassemia major, hemoglobin and RBC synthesis are defective or absent, thus from infancy onward, patients are dependent on frequent chronic blood transfusions for life. Over the course of their lives, patients with thalassemia major will receive hundreds to thousands of RBC units and as a result experience significant organ damage from iron deposition, particularly to the liver and heart. Life expectancy is significantly shortened

in thalassemia major, with the majority of deaths attributed to cardiac failure due to accumulated iron in the heart (7).

Sickle cell disease occurs primarily in individuals of African descent and is highly prevalent in the U.S. African-American population; approximately 1 in 12 African-Americans carry the sickle hemoglobin (HbS) gene trait, and 1 in 400 have homozygous (HbSS) or compound heterozygous SCD (HbSC, HbSβ-thalassemia, and others). In contrast to thalassemia, which is a quantitative defect in Hb and RBC synthesis, SCD is a qualitative defect in which the defective sickle hemoglobin molecule (HbS) is able to polymerize into long rods, distorting the RBC into an elongated sickle shape. Abnormal sickle RBCs become trapped in the microvasculature resulting in painful vaso-occlusive crises, stroke, acute chest syndrome, and splenic sequestration. Over time, repeated vaso-occlusive events and alteration of the blood vessel endothelium result in severe damage to lungs, cerebral vasculature, kidneys, and bone. RBC transfusion is critically important during acute, life-threatening events to rapidly decrease the percentage of circulating sickle RBCs. Patients who have multiple recurrent complications or who are found to be at risk for stroke require chronic transfusion therapy to maintain a low percentage of circulating sickle RBC and decrease the risk of subsequent complications. As in thalassemia major, SCD patients on chronic transfusion therapy receive RBC transfusions every 3 to 5 weeks; patients who have had a stroke due to SCD require chronic transfusions for life to prevent future strokes (8).

The use of RBC transfusions to treat SCD and thalassemia is complicated by alloimmunization to RBC antigens. In addition the major ABO blood type antigens, more than 250 minor RBC antigens have been identified (9). An individual's RBC

phenotype refers to the expression or absence of common antigens on the surface of the RBC. While antibodies to A and B major antigens form innately, alloimmunization to all minor RBC antigens only occurs via blood transfusion or fetal-maternal blood exposure. Typically RBC transfusions are specifically matched only for an individual's ABO and Rh(D) types, the most immunogenic RBC antigens, thus mismatch occurs for multiple minor RBC antigens. Despite minor RBC antigen-mismatched blood transfusions, alloimmunization is an uncommon event, occurring at a low incidence of 2.6 – 2.9% among all non-hemoglobinopathy patients receiving RBC transfusions (10, 11).

Patients with SCD or thalassemia have significantly higher rates of antibody formation in response to RBC transfusion. In adult SCD patients the prevalence of RBC alloimmunization ranges 18.6 – 35% (1, 2, 12-14) while pediatric patients who are likely to have received fewer phenotype-unmatched transfusions have a lower rate of 7.75 – 17.6% (15, 16). RBC alloimmunization occurs in 10 – 15% of thalassemia patients, though the incidence may be as low as 3.7 – 5.2% in patients receiving exclusively phenotype-matched RBC (3, 17, 18). The higher rate of alloimmunization in both patient groups may reflect a number of causes including a high number of lifetime transfusions (11), phenotype-unmatched blood (19), and the racial disparity with a blood donor population that is predominantly Caucasian (20, 21). Phenotype matching of RBC transfusions for even a few immunogenic minor RBC antigens (such as C, E, and Kell) significantly reduces the rate of alloimmunization and transfusion-related complications in SCD and thalassemia patients but is limited by the scarcity of phenotypically matched blood donors (19, 22-25). At many but not all tertiary care pediatric hospitals, prophylactic phenotype matching for all SCD patients has been standard practice for

approximately a decade; patients transfused prior to implementation of this practice received more phenotypically dissimilar RBC transfusions (24, 26-29).

Even with RBC minor antigen phenotype matching, it is apparent that some patients are prone to produce multiple alloantibodies, while other patients are immunologic "non-responders," despite exposure to highly immunogenic foreign antigens such as Rh(D) (15, 30). The genetic and environmental factors that determine a patient's predisposition to alloimmunization are not fully understood (31, 32). Interestingly, in thalassemia major, children who begin chronic transfusion therapy within the first 12-36 months of life have a much lower incidence of alloimmunization, suggesting exposure at an early age may induce tolerance to foreign RBC antigens (3, 18). Evidence from murine models suggests that splenectomy also may be a protective factor against alloimmunization (33). Ultimately however, it is still not known why some patients have strong alloantibody responses while others with similar transfusion exposures do not develop alloimmunization.

The main consequence of RBC alloimmunization is hemolytic transfusion reactions (34, 35). Although patients are screened for alloantibodies to prevent transfusion of incompatible blood, low-titer antibodies may fall below the limits of detection or new alloantibodies may develop during transfusion, in both instances resulting in hemolysis of the transfused RBC (14). Delayed hemolytic transfusion reactions (DHTR) typically are detected 3 to 21 days after transfusion and typically result in mild hemolytic anemia without significant morbidity, however in rare instances may be fatal (36, 37). Some patients with SCD are prone to more severe hyperhemolytic delayed transfusion reactions, a phenomenon termed the "sickle cell transfusion reaction

syndrome" (35). In hyperhemolysis, it is believed that uncontrolled hemolysis of the incompatible transfused RBC triggers further hemolysis of the patients' autologous RBC (34, 38, 39). This reaction is characterized by fever, diffuse pain, and unexplained anemia and reticulocytopenia with hemoglobin levels typically lower than pre-transfusion. Further RBC transfusion, even with phenotypically-similar RBC, typically worsens the hemolysis. Although the causes of hyperhemolytic transfusion reactions most commonly are attributed to RBC alloantibodies, an unusual feature of this syndrome is that detection of a causative RBC antibody may be delayed by weeks or may never be identified (37). In cases of hyperhemolysis in which causative RBC antibodies are not identified, other immunologic triggers such as HLA alloantibodies may be responsible (40, 41).

Blood transfusion exposes a recipient to many other foreign antigens recognized as "non-self" by the immune system. Human leukocyte antigens (HLA), also known as the major histocompatibility complex (MHC) are a group of several cell surface markers with incredible interracial and intra-racial genetic polymorphism. HLA are divided into two major classes of proteins that are recognized by the immune system as antigen-presenting molecules. Class I HLA consist of three unique antigens (HLA-A, HLA-B, and HLA-C) present on the surface of all nucleated cells except mature RBCs; class II HLA molecules (HLA-DR, HLA-DQ, HLA-DP) are present only on particular antigen-presenting white blood cells (WBC). Class II molecules are made up of an alpha and beta chain, each with significant genetic polymorphism, while class II HLA have polymorphism only in a single alpha chain. The genes encoding the six loci HLA have the greatest diversity in the entire genome, with over 1500 unique alleles identified to

date, playing a key role in the immune system's recognition of self and mediating immune rejection of non-self cells. The population frequency and the associations between HLA alleles have considerable differences among human races and populations (42).

Formation of alloantibodies directed to specific HLA antigens is commonly seen with exposure to foreign tissue (e.g. pregnancy and organ transplantation) or with transfusion of platelets or WBC. Although RBC transfusion typically does not result in HLA antibody formation, HLA exposure does occur. Although mature RBC are anucleate, trace numbers of class I HLA have been detected on the surface of RBC (43, 44), and HLA alloimmunization following RBC transfusion has been documented in SCD and non-SCD patients (45-47). Another source of HLA alloimmunization may be from exposure to the Bennett-Goodspeed antigens, a group of minor RBC antigens that are not detected on routine pre-transfusion screens. Bg antigens have nearly identical homology to specific HLA (HLA-B7, B57, B58, HLA-A2, A28, A68, A69) (48, 49). Although these antigens have been considered to be insignificant in most transfusions, patients with alloantibodies directed against these HLA may be at risk for hemolytic reactions if Bg+ RBCs are transfused (41, 45, 50). Lastly, HLA alloimmunization may occur as a result of exposure to donor WBC in transfused RBC units (46, 51). Although leukofiltration of blood products significantly reduces WBC exposure, it is not sufficient to completely eliminate exposure to class I and class II HLA or prevent HLA alloimmunization with RBC transfusion (52-54).

HLA alloantibodies mediate several adverse outcomes in terms of rejecting organ transplant allografts, stem cell grafts, platelet transfusions and perhaps RBC transfusions.

In solid organ transplantation, HLA antibodies are known to cause hyperacute allograft rejection and decrease overall survival time of the organ (55-58). In HSCT, HLA antibodies may result in rejection of HLA-mismatched grafts (59-61). Additionally, patients are dependent on multiple platelet transfusions for a minimum 2 to 4 week period during HSCT, and HLA alloimmunization may result in platelet transfusion refractoriness and serious hemorrhage (62). The importance of screening patients for the risk of rejection both prior to and after transplantation has led to the development of sensitive techniques for HLA antibody detection in the serum of patients who have had HLA exposure via transfusion or pregnancy. HLA Panel reactive antibody (PRA) is a measure of antibody reactivity against a broad range of HLA antigens found in the general population. High titers of HLA antibodies (>80% PRA) are a contraindication to receiving an organ transplant according to guidelines set by the United Network for Organ Sharing (UNOS) (63, 64). Methods that combine antibody binding assays with flow cytometric detection are the most sensitive and specific techniques available (65-67). Since SCD and thalassemia major patients have severe lifelong morbidity, shortened life expectancies, and may have severe renal or cardiac damage, organ transplantation and HSCT are reasonable treatment considerations. However, if SCD or thalassemia major patients have HLA alloimmunization from past transfusions, their chances of benefiting from life-saving transplantation is slim.

Although HLA antibodies may cause complications with innovative and life-saving therapeutic interventions in SCD and thalassemia patients, the incidence of HLA alloimmunization from RBC transfusion in SCD or thalassemia patients is unknown.

Study Rationale and Specific Aims

Understanding the baseline occurrence and predictors of HLA alloimmunization among multiply-transfused patients with SCD and thalassemia major is necessary for subsequent investigations of the roles that HLA alloantibodies may play in hyperhemolytic transfusion reactions and HSCT graft rejection in these patient populations. Since the prevalence of RBC alloimmunization is known to be significantly higher in SCD patients and thalassemia major patients as compared to other groups receiving multiple RBC transfusions, it is likely that the rate of HLA alloimmunization will also be greater. An association of RBC alloimmunization with HLA alloimmunization would further support the notion that individuals with alloimmunization to foreign blood antigens share common immunologic responder characteristics. Determination of HLA antibody specificity in these cohorts may lead to generation of new hypotheses regarding the role that HLA antibodies may play in transfusion reactions such as DHTR.

METHODS

Null Hypotheses

1. Multiply-transfused pediatric patients with SCD or thalassemia major will not have a prevalence of HLA alloimmunization equal to or greater than the prevalence of RBC alloimmunization.

2. In pediatric SCD patients, a documented history of current or past RBC antibodies (alloantibodies and/or autoantibodies) will not be associated with HLA alloimmunization.

3. In pediatric SCD patients, chronic transfusion therapy will not be associated with increased or decreased rate of HLA alloimmunization.

Study Design

This study is a cross-sectional observational study of HLA alloimmunization in two distinct cohorts of multiply-transfused pediatric patients: cohort 1 consists of homozygous (hemoglobin SS) SCD or severe compound heterozygous (hemoglobin S-β^0 thalassemia) SCD; cohort 2 is transfusion-dependent thalassemia major. Institutional review board approval for this investigation was granted by Emory University and Children's Healthcare of Atlanta (CHOA) for enrollment at two pediatric tertiary care hospitals within Atlanta: Egleston Children's Hospital (ECH) and Scottish Rite Hospital (SR) which combined serve approximately 600 to 700 hemoglobin SS and S-β^0 thalassemia SCD patients and 20 transfusion-dependent thalassemia major patients. Written informed consent was obtained from the legal guardian for patients under age 18 years; written assent was obtained from patients aged 11 to 17 years, and verbal assent was obtained from patients aged 6 to 10 years.

In cohort 1, patients were selected in a case-control fashion to assess the association of RBC antibodies with HLA alloimmunization. Case subjects were defined as those with RBC alloantibodies and/or autoantibodies ever detected on routine indirect antiglobulin testing. Control subjects were defined as those with no history of RBC antibodies. Case and controls were matched for chronic transfusion status.

As cohort 2 is a much smaller group of patients, the aim was to determine the population prevalence of HLA alloimmunization among pediatric thalassemia major patients receiving care from CHOA. All thalassemia major patients meeting inclusion and exclusion criteria were approached for study enrollment.

Patient Eligibility

Inclusions Criteria

(1) Diagnosis of hemoglobin SS or S-β^0 thalassemia SCD or diagnosis of thalassemia major (defined by lifetime dependence on chronic transfusions due to severely inadequate RBC production).

(2) Age $\geq$ 3 years-old and $\leq$ 21 years-old

(3) Three or more lifetime RBC transfusions

Exclusion Criteria

(1) Past or current pregnancy

(2) Past history of intravenous immunoglobulin (IVIG) infusion

(3) Past history of platelet transfusion

(4) Past history of bone marrow, stem cell, or solid organ transplantation

(5) Current fever, bacteremia, or sickle cell crisis

(6) Use of systemic corticosteroids within 3 weeks prior to enrollment

Subject Identification and Enrollment

Cohort 1:

At ECH and SR hospitals, CHOA provides specialized hematology care for approximately 1100 to 1200 children with homozygous (HbSS) and compound heterozygous SCD. Approximately 600 of these children have HbSS or Hb S-β^0 thalassemia SCD, the most severe genotypes in which children are most likely to require transfusions. A review of blood bank transfusion records for all children with SCD from 1999 to 2008 for ECH indicated that for children ages 3 to less than 10 years, 32% have received transfusion of 3 or more RBC units; for children ages 10 to less than 18 years, 58% have received 3 or more RBC units. Given this data, it was estimated that there were 75 children aged 3 to <10 years and 154 children aged 10 to 18 meeting inclusion criteria of hemoglobin diagnosis, age, and transfusions, for a total of 229 potentially eligible subjects. To identify potential case subjects, the blood bank databases of ECH and SR were searched for patients with record of RBC antibodies and request for sickle-negative RBC units. To identify potential control subjects, patients presenting to hematology clinic were screened for eligibility. Eligible patients were approached for study enrollment during their routine outpatient medical appointments.

Cohort 2:

Pediatric thalassemia major patients were identified from the clinic database and were approached for study enrollment when they presented for monthly chronic transfusion at a CHOA clinic.

HLA Antibody Detection Methods

Patient serum was screened for anti-HLA immunoglobulin G (IgG) alloantibodies using FlowPRA® Screening test (One Lambda, Inc., Canoga Park, CA), a flow cytometry-based test that screens patient serum against a panel of HLA antigens, enabling the simultaneous detection of class I and class II HLA antibodies (68). FlowPRA uses a panel of microparticles, each coated with 2 or 3 unique HLA molecules, representing all the common and many of the rare, clinically relevant HLA antigens and epitopes in the North American population (69). The percentage of microparticles that react with antibody in the patient's serum, quantitated by flow cytometry, is termed the panel reactive antibody (PRA) activity, estimating the proportion of the population to which the patient is alloimmunized.

For the FlowPRA assay, serum was incubated with non-fluorescent microparticles coated with purified class I HLA (FlowPRA I beads) and with class II HLA-coated microparticles that have a fluorescent emission at 580 nm (FlowPRA II beads). Incubated beads were stained with fluorescein isothiocyante (FITC)-conjugated goat anti-human IgG (Fe fragment specific; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Particle fluorescence was analyzed on a FACScan (Becton-Dickinson, Mountain View, CA, USA) flow cytometer. Fluorescent FlowPRA II beads were detected on the FL2 channel; emission from FITC-conjugated antibody, indicating the presence of HLA antibodies, was detected by FL1 channel shift. Plots of FlowPRA I and FlowPRA II beads were isolated based by forward scatter (bead size) versus FL2 emission. FL1 emission histograms for FlowPRA I and II beads indicated the percentage of beads bound

with HLA antibodies from patient serum (% PRA). Results were considered positive when ≥5% of beads showed FL1 channel shift emission. Positive FlowPRA screening assays were reflexed to LabScreen® Single Antigen Antibody Detection (One Lambda, Inc., Canoga Park, CA) for result verification and identification of the specific HLA antibodies. This confirmatory assay employs fluorescent beads with unique FL2 channel shifts, each coated with a single HLA allele.

FlowPRA is the most sensitive technique currently available to detect HLA antibodies. Sensitivity is 20% to 30% higher than antiglobulin-enhanced lymphocyte cytotoxicity or ELISA assays (61, 70). False reactivity with the assay is presumed to be near zero.

Predictor and Outcome Variables

The primary outcome of interest was detection of HLA alloantibodies by FlowPRA® and LabScreen® assays. For patients with SCD (cohort 1), the primary predictor variable of interest was prior detection of antibodies to RBC antigens, either alloantibodies or autoantibodies. Other predictor variables were the age of the patient, chronic transfusion therapy (current, past, or never), number of days from last RBC transfusion, estimated lifetime number of RBC units transfused (less than 25 units, 25 to <50 units, 50 to <100 units, 100 or more units), and splenectomy. Age and number of days from last transfusion were assessed as continuous and as categorical variables (age <13 or ≥ 13 years, last transfusion < 90 or ≥ 90 days prior). Chronic transfusion therapy was defined as treatment with scheduled RBC transfusions every 3 to 5 weeks for prevention of SCD complications. Current chronic transfusion therapy was defined as

receiving transfusions for at least the three months prior to study enrollment; past chronic transfusion therapy was defined as any chronic transfusion therapy that had been discontinued ≥ 3 months prior to study enrollment. Chronic transfusion therapy was analyzed as a potential confounder of the relationship between RBC and HLA antibodies since theoretically repeated exposure to foreign blood antigens could increase the likelihood of HLA alloimmunization or conversely could induce immunologic tolerance and decrease the likelihood of HLA alloimmunization. Since the effect of chronic transfusion therapy on HLA alloimmunization was unknown, cases and controls were matched for chronic transfusion status.

For patients with thalassemia major (cohort 2), the anticipated patient sample size was too low to identify predictors of HLA alloimmunization. The main aim in cohort 2 was to determine the overall frequency of HLA alloimmunization. History of RBC alloimmunization was recorded to determine if an association existed with HLA alloimmunization.

Sample Size

Based on previous studies of anti-platelet and anti-HLA antibodies in SCD and non-SCD patients receiving non-leukoreduced RBC transfusions, it was assumed that SCD control subjects in this study would have a HLA alloimmunization rate of at least 20% (46, 47, 51). A rate of HLA alloimmunization of 50% or greater in case subjects was judged to be clinically significant enough to potentially alter medical decision making. Based on this judgement and selecting case and control subjects in a 1:1 ratio, the study was powered to detect a case-control odds ratio of 4. To demonstrate this

difference with 80% power and 95% confidence (α=0.05), a total sample size of 76 patients (38 cases and 38 controls) was required.

Analysis for Cohort 1

In the cohort of SCD patients, HLA alloimmunization was assessed in two groups: children with RBC antibodies (cases) to those without RBC antibodies (controls). To assess for confounding in selection of cases and controls, characteristics of case and control patients were compared by chi-square or Fisher exact test for categorical variables and t-test for continuous variables. The frequency of HLA alloimmunization in case and control groups was compared by chi-square test, and a crude odds ratio assessing the relationship of RBC antibodies with HLA alloantibodies was calculated. HLA alloimmunization rates were also compared for subjects with and without RBC alloantibodies and for subjects with and without RBC autoantibodies. The frequency of the other co-variates in subjects with and without HLA alloimmunization was compared by chi-square comparison. Stratified odds ratios for the effect of RBC antibodies on HLA alloimmunization were calculated within each stratum of categorical variables, and the Breslow-Day test for heterogeneity of effect was used to detect interaction with RBC antibodies using an α significance level of 0.05. To control for confounding, Mantel-Haenszel adjusted odds ratios were calculated.

Multivariate logistic regression modeling was performed using all predictor variables. Parameter estimates and odds ratios for each variable were calculated by maximum likelihood estimates analysis, and statistical significance was determined by Wald chi-square test with an α significance level of 0.05. Goodness of fit to the model

was assessed by Hosmer and Lemeshow test. Collinearity of variables was assessed by calculation of variance decomposition proportions (VDPs) and conditional indices.

To explore the interactions of co-variates with RBC antibodies suggested by stratified univariate analysis, logistic regression was performed including terms for the interaction of RBC antibodies with current chronic transfusion therapy and with past chronic transfusion therapy. Statistical significance of the interaction terms was assessed by Wald chi-square test. The likelihood ratio test was used to compare the models with and without interaction terms.

Due to age bias in selection of case and control subjects, conditional logistic regression matching cases and controls by age was performed with proportional hazards regression analysis; hazards ratios for each predictor variables were calculated.

RESULTS

Patient Accrual

Children with SCD presenting to hematology clinic at ECH and SR for routine care from September 2005 to November 2008 were periodically reviewed to identify eligible study participants. Ninety-six eligible patients/guardians (39 cases, 58 controls) were approached for study participation; of these 23 (24%) declined and 73 consented for study testing (Table 1). Of the 96 patients approached, 49 (51.0%) were on chronic transfusion therapy, and 38 had RBC antibodies (39.6%). Refusal rate was 18.4% among those on chronic transfusions and 29.8% among those not on chronic transfusions (chi-square p=0.190). Refusal rate was 21.1% among those with RBC antibodies and 25.8% among those without RBC antibodies (chi-square p=0.589). Patients who declined participation tended to be younger with a mean age of 9.9 years (SD 4.4 years) versus a mean age of 12.8 years (SD 4.3 years) for enrolled study subjects (pooled t-test p=0.005).

In the entire cohort of thalassemia major patients at CHOA, 17 met eligibility criteria and were approached for study participation. All 17 children participated in the study.

Result Validity

Repeat sampling of subjects for validation of results over time was not performed, however 9 subjects (6 SCD, 3 thalassemia) had repeat HLA FlowPRA testing performed for clinical purposes either before or after study enrollment. Five patients had negative FlowPRA tests on both testing dates, with a mean of 383 days between tests (range 0 - 711 days). Three patients had positive FlowPRA assays on both dates, tested a mean of

1318 days apart (range 633 – 2258 days); two of these patients showed an increase in PRA percentage over time, while one patient showed a decrease in PRA. Only one patient had positive and negative results at two different time points. This patient had a positive PRA (class I, 14%) detected during a severe DHTR prior to study but a negative PRA when tested on study 638 days later; the patient had received 20 subsequent RBC transfusions between the positive and negative PRA tests.

Cohort 1: SCD Results

Of 73 SCD participants, 30 (41.4%) had previously detected RBC antibodies and 43 (58.9%) did not have RBC antibodies. Within the group of patients with RBC antibodies, 21 (28.8%) had RBC alloimmunization and 15 (20.5%) had RBC autoantibodies. Six patients (8.2%) had both RBC alloantibodies and autoantibodies. The characteristics of case and control groups are compared in Table 2. Patients with RBC antibodies tended to be older (14.5 ± 3.0 years) than those without RBC antibodies (11.7 ± 4.7 years) (t-test, p=0.003). The RBC antibody group had a greater proportion of patients with higher numbers of lifetime transfusions, although this trend was not statistically significant (chi-square p=0.397). Other co-variates were similar between the case and control groups. Chronic transfusion status was nearly equally matched between case and control groups (50% of case patients on chronic transfusions, 58% of control patients on chronic transfusions, chi-square p=0.492).

Overall, HLA alloimmunization was detected in 25 (34.3%) SCD patients. Twenty-two patients had antibody directed against class I HLA alone, one patient had antibody to class II HLA alone, and 2 patients had antibodies to both class I and class II

HLA. HLA PRA results are summarized in Table 3. Among the 24 samples with class I HLA antibodies, the mean PRA was 52% (range 3 – 99%), indicating that in most patients, serum antibodies were reactive against over half the panel of class I antigens. Among the 3 patients with class II HLA antibodies, mean PRA was 47% (range 18 – 99%).

Table 4 shows the association of RBC antibodies with HLA alloimmunization; patients with RBC antibodies were further categorized into those with RBC alloantibodies and those with autoantibodies. RBC antibodies were highly associated with HLA alloimmunization (OR 4.32 [1.55 – 12.05]). RBC autoantibodies had a stronger association with HLA alloimmunization (OR 3.94 [1.21 – 12.85]) than RBC alloantibodies (OR 2.99 [1.04 – 8.56]). Table 5 shows the association of all co-variates with HLA alloimmunization.

In Table 6, stratified analysis assessing interaction and confounding effects of co-variates on the relationship between RBC antibodies and HLA alloimmunization is shown. Interaction exists between RBC antibodies and chronic transfusion therapy, as shown by Breslow-Day test. The sub-stratum odds ratios suggest that past chronic transfusion therapy (but not current chronic transfusion therapy) opposes the association of RBC antibodies with HLA alloimmunization. The association of RBC and HLA antibodies is greatest among subjects with no history of chronic transfusion exposure. No other co-variates showed evidence of interaction or confounding.

Multivariate logistic regression analysis including all predictor variables is shown in Table 7. VDP matrix had a maximum conditional index of 10.22, indicating no significant collinearity between the predictor variables. In multivariate analysis, the only

factor that significantly increased the odds of HLA alloimmunization was RBC antibodies (p=0.032). Past or current chronic transfusion therapy showed a trend towards lower odds of HLA alloimmunization, however the effects were not significant. Splenectomy also showed a trend toward lower odds of HLA alloimmunization that did not reach significance at alpha=0.05 level. The number of lifetime transfusions again was not a statistically significant predictor, however there was a trend towards the highest odds of HLA alloimmunization for individuals in the 50-100 lifetime unit range (estimated coefficient 1.99, OR 0.87 – 61.0) but not for individuals who had exceeded 100 lifetime transfusions. When lifetime transfusions were categorized into two groups (less than 50 or ≥ 50 lifetime units), the effect of having 50 or more lifetime RBC transfusions was close to statistical significance, with an estimated coefficient of 1.74 (S.E. 0.89) and OR 5.72 (95% C.I. 0.99 – 32.9, p=0.051).

Introduction of interaction terms into multivariate analysis (Table 8) showed significant interaction between RBC antibodies and past chronic transfusion therapy (p=0.011) but not with current chronic transfusion therapy (p=0.304), suggesting that past exposure to chronic transfusions may be more protective against HLA alloimmunization than ongoing exposure. Likelihood ratio test contrasting the interaction and non-interaction models showed a significant difference between the two models (χ^2 LRT p=0.014). In age-matched conditional logistic regression analysis, shown in Table 9, only RBC antibodies remained a significant predictor of HLA alloimmunization, with a hazard ratio of 5.04 (95% C.I. 1.01 – 25.2, p=0.049).

Cohort 2: Thalassemia major results

Of 17 participants with thalassemia major, HLA alloimmunization was detected in 8 (47.1%). Two patients had antibody to class I HLA alone; 2 patients had antibody to class II HLA alone; 4 patients had alloimmunization to class I and class II HLA. Mean PRA for class I HLA antibodies was 77% ± 26%; mean PRA for class II HLA antibodies was 48% ± 22%, indicating a high likelihood of antibody cross-reactivity with potential random population donors. HLA PRA results are summarized in Table 10.

Table 11 compares patient characteristics for thalassemia major patients with and without HLA alloimmunization. Three patients (17.6%) had RBC alloantibodies. HLA alloimmunization was detected in 2 (67%) patients with RBC antibodies and 6 (43%) patients without RBC antibodies (Fisher's exact p=0.5765). Age and number of days from last RBC transfusion were not significantly different between patients with and without HLA antibodies.

HLA Antibody Specificity Analysis

Among the 25 SCD patients with HLA alloimmunization, 15 (60%) had antibody against at least one HLA antigen with close homology to the RBC Bennett-Goodspeed (Bg) antigens. Ten patients had antibody to HLA-B7 (Bg^a), 6 patients had antibody to HLA-B57/B58 (Bg^b), and 11 patients had antibody to HLA-A2/A68/A69 (Bg^c). Delayed hemolytic transfusion reactions (DHTR) were documented in 3 of the 15 patients with Bg-HLA antibodies (20%) and 4 of the 58 patients without Bg-HLA antibodies (7.4%) (OR 3.38 [95% C.I. 0.67 – 17.1], Fisher's exact test p=0.148).

Among the 9 thalassemia patients with HLA alloimmunization, 8 samples were adequate for HLA antibody specificity testing. Among the 8 thalassemia patients tested,

4 (50%) had antibodies to antigens homologous to the Bg antigens (4 with anti-Bg^a, 2 with anti-Bg^b, 2 with anti-Bg^c).

DISCUSSION

HLA alloimmunization is an under-recognized phenomenon that may have serious consequences in medical conditions that are primarily treated by transplantation or transfusion of allogeneic organs, stem cells, or blood products. Only select patient populations, such as individuals awaiting renal or cardiac transplantation or individuals awaiting HSCT for aplastic anemia, are typically screened for HLA alloimmunization (57, 71). However, despite the fact that SCD and thalassemia patients who have had numerous RBC transfusions also may be at risk, clinicians do not routinely monitor for HLA alloimmunization in these patients. Thus the frequency and predictors of HLA alloimmunization are unknown. As HSCT, which has cured hundreds of patients with SCD and thalassemia major, becomes a more common therapeutic consideration for these patients, recognition of HLA alloimmunization is important in identifying individuals at risk for poor HSCT outcome due to graft rejection or platelet transfusion refractoriness. Furthermore, identifying individuals at risk for HLA alloimmunization and modifying transfusion practices early to prevent this occurrence is extremely important if HSCT is to be offered to more patients in the future. Lastly, there is significant interest in the scientific community regarding the role that HLA antibodies might play in initiating bystander hemolysis, a severe transfusion reaction that is not uncommon in pediatric SCD patients (37) and often has no identifiable cause. A causal association between HLA antibodies and bystander hemolysis is merely speculative (although supported by some *in vitro* investigations), thus gathering data on the frequency of HLA antibodies in healthy SCD and thalassemia patients is a preliminary step to further investigations of the

role that HLA antibodies may play during hemolytic transfusion reactions in these patients.

A significant finding in this study is the overall high frequency of HLA alloimmunization in pediatric thalassemia major and multiply-transfused SCD patients. Over one-third of SCD patients and nearly half of all pediatric thalassemia patients in this cross-sectional sampling demonstrated HLA alloimmunization. This is significantly greater than the rate of RBC alloimmunization typically found in pediatric SCD and thalassemia patients. Also of surprise was the high frequency of HLA antibodies directed against antigens known to occur on the surface of RBC (the Bennett-Goodspeed antigens). This analysis suggests that a SCD or thalassemia patient with HLA alloimmunization has a 50-60% probability of having antibodies that may be cross-reactive with RBC minor antigens. This finding may suggest that a significant proportion of HLA alloimmunization is due to direct exposure to RBC surface antigens rather than to trace amounts of WBC in the transfused blood units. However the identification of these antibodies alone does not predict an adverse reaction such as DHTR, and further studies are needed to determine if antibodies to Bg antigens are harmful to patients receiving RBC transfusions.

For the thalassemia major cohort, the rate of HLA alloimmunization represents the true prevalence for the pediatric population in Atlanta. While the number of patients was too small to draw conclusions regarding factors associated with HLA alloimmunization, it is interesting to note that children with HLA alloimmunization tended to be older (age 14.4 years vs. 10.8 years) and also tended to have received their last RBC transfusion more recently (23 days vs. 28 days) than those without HLA

alloimmunization. The difference in age may reflect the fact that older patients have had more lifetime RBC transfusion exposures and perhaps may have been exposed to more non-leukocyte-depleted RBC transfusions in earlier years. The difference in timing from last transfusion probably does not reflect loss of antibody detection over such a short time period, however one could speculate that the presence of HLA antibodies results in shorter survival time of transfused RBC, requiring a patient to receive transfusion on a more frequent basis. This interesting speculation bears further prospective investigation in these patients.

In the SCD cohort, patients were intentionally selected to test hypotheses regarding risk factors for HLA alloimmunization. Thus the frequency of HLA antibodies observed in this cohort does not reflect the population prevalence, and a direct comparison cannot be made with the prevalence observed in thalassemia patients. Nevertheless, even in the group of patients found to be at lower risk (i.e. those with no RBC antibodies), the rate of HLA alloimmunization exceeded 20%, a frequency high enough to justify routine screening of all patients who have had multiple lifetime RBC transfusions. The high percentage PRA (>50%) seen in the majority of the children with HLA alloimmunization is worrisome in that the PRA reflects the proportion of donors in the population with whom a patient may be incompatible due to preformed HLA antibodies. Thus, the likelihood of rejecting a transplanted organ, stem cells, or platelet donation is high. It also is of interest to note that alloimmunization to class II HLA was rare among SCD patients, occurring in only 3 (4%) children, all of whom had RBC antibodies, as compared to a relatively high occurrence of 35% in thalassemia major children.

Perhaps even more significant than the overall high frequency of HLA alloimmunization observed in this study is the finding of some individuals who have resisted HLA alloimmunization despite numerous and recent exposures to foreign blood antigens. As shown by both multiple logistic regression and age-matched conditional logistic regression, RBC antibodies (both alloantibodies and autoantibodies) were strongly associated with HLA alloimmunization. This association does not suggest a causal link from one to another; rather it might suggest that some individuals inherently are at greater risk for alloimmunization to a broad range of foreign blood. The concept of an immunologic phenotype for alloantibody hyper-responders versus non-responders is clinically observed for RBC alloimmunization. Patients who have made one RBC antibody are much more likely to develop additional RBC alloantibodies with subsequent transfusions, while other patients may never develop RBC antibodies despite repeated exposures to minor antigen mismatched RBCs (31, 32). What remains unclear are the factors that shape an individual's responder status or immunologic phenotype. It is extremely valuable to note that HLA alloimmunization is associated with RBC allo/autoimmunization in order to more precisely define a responder phenotype and to subsequently identify clinical features that may predict future antibody responsiveness.

In this analysis we sought to define three HLA antibody responder phenotypes in SCD patients that were independent of past transfusion exposures: (1) patients who have antibody to both HLA and RBC, (2) patients with HLA antibodies without RBC antibodies, and (3) patients without HLA antibodies. Due to the limited sample size of this study, the predictor variables other than RBC antibodies that were analyzed did not show statistically significant associations with HLA alloimmunization, however some

important trends were noted. What was most striking in the data is that some individuals were highly prone to the formation of antibodies to multiple foreign antigens, demonstrating high HLA PRA and multiple RBC antibodies, while other individuals were seemingly tolerant to foreign antigens, despite similarly high burdens and recent timing of transfusions. Among patients with RBC antibodies, exposure to past chronic transfusions decreased the likelihood of an antibody response to foreign HLA, a finding that was significant in stratified analysis. Although it seems contradictory that more exposures to foreign HLA would result in less alloimmunization, this finding is consistent with past studies showing that children with thalassemia major who begin chronic transfusions before the age of 12 months have a significantly lower rate of RBC alloantibody formation than those who start later in life (18). From this observation one might hypothesize that repeated doses of foreign HLA are more likely to induce immunologic anergy rather than an antibody response. Patients currently on chronic transfusion therapy did not show this same trend towards less HLA alloimmunization. Further investigations are needed to explore this interaction of chronic transfusion exposure and alloimmunization to transfused antigens.

Another significant finding was the trend towards lower rates of HLA alloimmunization among splenectomized patients. This finding is not unexpected, since the spleen is primarily responsible for stimulating immunologic antibody responses to bloodstream antigens (72), however the association of splenectomy with decreased alloimmunization had previously been noted only in murine models (33). Thus its demonstration in human subjects is noteworthy.

There were several limitations to this study that are worth noting. Prior studies have shown that HLA antibody detection may appear or disappear as time from transfusion elapses (51, 73). Due to the cross-sectional design of the study, the incidence of new HLA antibody formation as well as the loss of HLA antibody over time could not be determined. In this analysis, eight of nine subjects had consistent HLA antibody results at later time points. This is not a large enough validation set to draw any conclusions regarding the consistency of HLA antibody testing, thus it remains important for clinicians to recognize that patients with negative HLA PRA may still be at risk for adverse sequelae of HLA alloimmunization.

The second major limitation in the SCD cohort was subject selection bias to due to patient refusal. Nearly one-fourth of SCD patients approached declined to participate due to a variety of reasons including the extra loss of blood (2-3 milliliters), trauma of phlebotomy in a younger child, and a lack of interest in the research outcome. Case and control groups were not balanced in terms of age, however efforts to more appropriately age-match the groups were hindered by the fact that the guardians of younger patients were more likely to refuse study participation (Table 1). Those who agreed to participate were more likely to be on chronic transfusion therapy ($p=0.19$), also introducing bias into the results and allowing fewer conclusions to be drawn about patients not on chronic transfusions. Definitive conclusions regarding the effects of splenectomy and lifetime transfusion exposures also cannot be made due to the limited sample size of the study.

This analysis represents the first description of HLA alloimmunization specifically in SCD patients and in pediatric thalassemia major patients transfused in the U.S. FlowPRA, a highly sensitive technique initially developed for the identification of

HLA antibodies in transplant patients, reflects the number of antigens to which a patient is alloimmunized and the likelihood that a patient will react against a random population donor. The most important findings from this study were not only the high frequency of alloimmunization and the broad range of antigen reactivity detected in SCD and thalassemia patients but also the absence of alloimmunization in a substantial number of patients who had multiple and recent RBC transfusions. Exposure to foreign antigen alone does not result in an immunologic response; rather other factors such as early age of exposure, splenic function, and genetics may play roles in determining antibody response. The association of RBC antibodies with HLA antibodies implies that individuals have an underlying predisposition towards or against alloimmunization in general. Larger scale prospective studies are needed to identify additional risk factors and underlying causes of HLA alloimmunization in SCD and thalassemia patients, so that ultimately physicians can attempt to modify transfusion therapy for those at greatest risk of alloimmunization.

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TABLES

Table 1: Characteristics of 96 SCD patients approached for study participation.

	Agreed to Participation (n=73)	**Declined Participation (n=23)**	**Total (n=96)**	**p-value**
Age, years *	12.8 ± 4.3	9.9 ± 4.4	12.1 ± 4.5	0.005
RBC Antibodies – no. (%)	30 (41.1)	8 (34.8)	38 (39.6)	0.589
On chronic transfusion therapy – no. (%)	40 (54.8)	9 (39.1)	49 (51.0)	0.190

* mean ± SD

Table 2: Case-Control Comparisons of SCD Patient Characteristics

	RBC Antibody Positive (n=30)	**RBC Antibody Negative (n=43)**	**Overall (n=73)**	**p-value**
Age (years)*	14.5 ± 3.03 (9.1 – 19.4)	11.7 ± 4.74 (3.9 – 20.1)	12.85 ± 4.33	0.0028
Last RBC transfusion (days)*	234 ± 446 (21 – 2265)	313 ± 551 (12 – 2280)	281 ± 509	0.5185
Lifetime transfusions – no. (%)				
<25	8 (26.7)	20 (46.5)	28 (38.4)	0.3970
25-<50	4 (13.3)	4 (9.3)	8 (20.0)	
50-<100	11 (36.7)	12 (27.9)	23 (31.5)	
>=100	7 (23.3)	7 (16.3)	14 (19.2)	
Chronic Transfusions				
Never	7 (23.3)	10 (23.3)	17 (23.3)	0.6915
Past	8 (26.7)	8 (18.6)	16 (21.9)	
Yes	15 (50.0)	25 (58.1)	40 (54.8)	
Splenectomy				
No	25 (83.3)	33 (76.7)	58 (79.5)	0.493
Yes	5 (16.6)	10 (23.3)	15 (20.5)	

* mean ± SD (range)

Table 3: HLA antibody PRA results in SCD patients.

PRA = Panel Reactive Antibody. All patients with class II HLA antibody had RBC antibodies.

HLA Antibody	**Frequency – no. (%)**	**% PRA (mean ± SD)**	**% PRA range**
Class I	24 (32.9)	52 ± 36	3 – 99
Class II	3 (4.1)	47 ± 45	18 – 99

Table 4: Association of RBC allo- and autoantibodies with HLA alloimmunization in SCD

	HLA Antibody Positive No. (%)	HLA Antibody Negative No. (%)	OR (95% C.I.)	Chi-square p-value
RBC Alloantibodies				
Positive (n=21)	11 (52.4)	10 (47.6)	2.99	0.0380
Negative (n=52)	14 (26.9)	38 (73.1)	(1.04 – 8.56)	
RBC Autoantibodies				
Positive (n=15)	9 (60.0)	6 (40.0)	3.94	0.0184
Negative (n=58)	16 (27.6)	42 (72.4)	(1.21 – 12.8)	

Table 5: Associations of patient characteristics and transfusion history with HLA alloimmunization in SCD

	HLA Antibody Positive No. (%)	HLA Antibody Negative No. (%)	OR (95% C.I.)	Chi-square p-value
RBC Antibodies				
Positive (n=30)	16 (53.3)	14 (46.7)	4.32	**0.0041**
Negative (n=43)	9 (20.9)	34 (79.1)	(1.55 – 12.1)	
Age				
≥ 13 yrs (n=36)	17 (47.2)	19 (52.7)	3.24	**0.0212**
< 13 yrs (n=37)	8 (21.6)	29 (78.4)	(1.17 – 9.00)	
Last RBC transfusion				
≥ 90 days (n=25)	9 (36)	16 (64)	1.13	0.8198
< 90 days (n=48)	16 (33)	32 (67)	(0.41 – 3.10)	
Chronic Transfusions				
Never (n=17)	6 (35.3)	11 (65.7)	n/a	0.9335
Past (n=16)	6 (37.5)	10 (62.5)		
Yes (n=40)	13 (32.5)	27 (67.5)		
Lifetime RBC Transfusions				
< 25 (n=28)	7 (25.0)	21 (75.0)	n/a	0.1097
25 - <50 (n=8)	1 (12.5)	7 (87.5)		
50 - <100 (n=23)	12 (52.2)	11 (47.8)		
≥ 100 (n=14)	5 (35.7)	9 (64.3)		
Lifetime RBC Transfusions				
< 50 (n=36)	8 (22.2)	28 (77.8)	2.98	**0.0327**
≥ 50 (n=37)	17 (45.9)	20 (54.1)	(1.08 – 8.23)	
Splenectomy				
No (n=58)	23 (39.7)	35 (60.3)	0.23	0.0555
Yes (n=15)	2 (13.3)	13 (86.7)	(0.05 – 1.14)	

Table 6: Stratified analysis for the effect of RBC antibodies on HLA alloimmunization

Variable	Individual Strata Odds Ratios				OR (MH)*	OR 95% CI	Breslow-Day † p-value
Age	≥13 (n=36) OR=2.52		<13 (n=37) OR=6.39		3.555	1.24 – 10.15	0.3969
Last Transfusion	≥90 days (n=25) OR=10.50		<90 days (n=48) OR=2.83		4.21	1.52 – 11.65	0.2593
Splenectomy	Yes (n=15) OR=2.25		No (n=58) OR=4.69		4.29	1.50 – 12.28	0.6516
Lifetime Transfusions	< 50 units (n=36) OR 11.0		≥50 units (n=37) OR 2.14		3.74	1.33 – 10.55	0.1499
	<25 15.0	25-<50 1.33	50-<100 2.45	≥100 1.88	4.18	1.42 – 12.2	0.4275
Chronic Transfusions	Yes (n=40) OR=7.875	Past (n=16) OR=0.333	No (n=17) OR=22.5		n/a	n/a	**0.0130**

* Maentel-Hanszel adjusted odds ratio
† Breslow-Day test for interaction with RBC alloimmunization

Table 7: Prediction of HLA alloimmunization in SCD patients: non-interaction logistic regression model

Variable	Estimated Coefficient	Estimated Standard Error	Coeff / S.E.	Odds Ratio Estimates	95% Wald C.I.	Wald χ^2 p-value
RBC Antibody	1.31	0.61	2.14	3.69	1.12 – 12.2	**0.032**
Age ≥ 13	1.19	0.73	1.62	3.28	0.78 – 13.8	0.105
Units 25-50	-0.33	1.42	-0.23	0.72	0.05 – 11.6	0.818
Units 50-100	1.99	1.08	1.84	7.31	0.87 – 61.0	0.066
Units ≥100	0.65	1.24	0.53	1.92	0.17 – 21.8	0.599
Current Chronic Transfusions	-0.66	1.30	-0.51	0.52	0.04 – 6.63	0.611
Past Chronic Transfusions	-1.05	1.22	-0.86	0.35	0.03 – 3.85	0.391
Last Transfusion	2.35×10^{-4}	7.38×10^{-4}	0.32	1.00	0.999 – 1.002	0.750
Splenectomy	-1.53	0.91	1.68	0.22	0.04 – 1.29	0.093

RBC Antibody = RBC alloantibody or autoantibodies previously noted
Age = Age of patient (1 = ≥13 years; 0 = <13 years)
Units 25-50 = Lifetime number of RBC transfusions (1= 25 to <50 units; 0 = other number)
Units 50-100 = Lifetime number of RBC transfusions (1 = 50 to <100 units; 0 = other number)
Units ≥100 = Lifetime number of RBC transfusions (1 = ≥100 units; 0 = <100 units)
Current Chronic Transfusions: (1 = on chronic transfusion therapy; 0 = not on chronic transfusion therapy)
Past Chronic Transfusions: (1 = previously on chronic transfusions; 0 = currently or never on chronic transfusions)
Last Transfusion = Days since last RBC transfusion (1 = ≥90 days; 0 = <90 days)
Splenectomy = History of surgical splenectomy (1 = yes; 0 = no)

Hosmer and Lemeshow Goodness of Fit test: χ^2=8.99, p=0.3434

Table 8a: Prediction of HLA alloimmunization in SCD patients: Logistic regression model with interaction

Variable	**Estimated Coefficient**	**Estimated Standard Error**	**Coeff / S.E.**	**Wald χ^2 p-value**
RBC Antibody	3.79	1.71	2.22	0.026
Age ≥ 13	1.23	0.81	1.53	0.125
Units 25-50	-0.53	1.49	-0.35	0.724
Units 50-100	2.03	1.12	1.79	0.074
Units ≥100	0.38	1.33	0.29	0.773
Current Chronic Transfusions	0.88	1.98	0.44	0.658
Past Chronic Transfusions	2.02	1.82	1.11	0.267
Last Transfusion	7.19×10^{-4}	1.04×10^{-3}	0.69	0.488
Splenectomy	-1.65	0.99	-1.67	0.095
RBC Ab * Current Chronic Tx	-1.97	1.91	-1.03	0.304
RBC Ab * Past Chronic Tx	-5.31	2.08	-2.55	**0.011**

Hosmer and Lemeshow Goodness of Fit test: χ^2=6.44, p=0.5983

Table 8b: Association of RBC antibodies and HLA alloimmunization in SCD, stratified by chronic transfusion status

	Chronic Transfusion Therapy		
	Current (n=40)	**Past (n=16)**	**Never (n=17)**
RBC antibodies – Odds Ratio (95% CI)	6.17 (1.05 – 36.3)	0.22 (0.02 – 2.68)	44.1 (1.56 – 1247.5)

Table 9: Age-matched conditional logistic regression model of HLA alloimmunization

Variable	Estimated Coefficient	Estimated Standard Error	Coeff / S.E.	Hazard Ratio	95% Hazard Ratio C.I.	χ^2 p-value
RBC Antibody	1.62	0.82	1.97	**5.05**	**1.01 – 25.2**	**0.049**
Units 25-50	-0.31	1.42	-0.22	0.73	0.05 – 11.9	0.827
Units 50-100	1.68	1.11	1.51	5.35	0.60 – 47.4	0.132
Units ≥100	0.92	1.25	0.74	2.52	0.22 – 29.4	0.461
Current Chronic Transfusions	-0.64	1.41	-0.46	0.53	0.03 – 8.35	0.649
Past Chronic Transfusions	-0.99	1.21	-0.82	0.37	0.03 – 3.99	0.414
Last Transfusion	1.46×10^{-4}	9.28×10^{-4}	0.16	1.00	0.998 – 1.002	0.025
Splenectomy	-1.27	1.01	-1.25	0.28	0.04 – 2.05	0.211

Table 10: HLA antibody PRA results in 17 thalassemia major patients

HLA Antibody	Frequency – no. (%)	PRA (mean ± SD)	PRA range
Class I	6 (35.3)	77% ± 26%	40 – 96%
Class II	6 (35.3)	48% ± 22%	23 – 75%

Table 11: Characteristics of Thalassemia major patients

	HLA Antibody Positive n=8 (47%)	HLA Antibody Negative n=9 (53%)	Overall	p
RBC Antibodies – no. (%) Yes No	 2 (25) 6 (75)	 1 (11) 8 (89)	 3 (17.6) 14 (82.4)	0.5765†
Age *	14.4 ± 3.1 (8.5 – 17.9)	10.8 ± 2.7 (4.6 – 17.1)	12.5 ± 4.3 (4.6 – 17.9)	0.0877
Time since last transfusion (days)*	23 ± 5.4 (15 – 28)	28 ± 5.4 (19 – 35)	25.6 ± 5.8 (15 – 35)	0.0756

* mean ± SD (range)
† Fisher's exact test