Red cell alloimmunization is associated with development of autoantibodies and increased red cell transfusion requirements in myelodysplastic syndrome

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**Supplementary Information**

### Supplementary Methods

Bone marrow (BM) morphology was re-assessed, if required, by independent hematopathologists. BM cytogenetics were categorised according to IPSS-R criteria. MDS subtypes were reclassified according to the WHO 2016 classification.

Pretransfusion testing was performed as per laboratory policies. Pretransfusion testing blood samples were submitted to the hospital’s transfusion laboratory before each transfusion including ABO/RhD typing and antibody screening. Antibody screening was performed using a 3-cell screen panel and the indirect antiglobulin technique (IAT) using the DiaMed Microtyping System. A positive antibody screen would be further investigated by testing patient plasma against an extensively phenotyped 11-cell panel and an auto-control (using patient’s RBCs). A direct antiglobulin test (DAT; DiaMed Microtyping System) was performed on those samples with a positive auto control to test for the presence of RBC-bound IgG or complement (C3d) or both. In the case of DAT positive for IgG (± C3d), elution studies were performed using acid glycine. The eluate was tested against an 11-cell panel by IAT using the DiaMed System. Elution results were reported as non-reactive, reactive but non-specific (reactive with all panel cells) or reactive and antibody specific. Transfusion reaction data including febrile non-hemolytic (FNHTR), AHTR and DHTR were accessed from the hospital’s transfusion unit records.

### Statistical analysis

The cumulative incidence of alloimmunization was analysed by competing-risks regression using the Fine and Gray method. Presence of alloantibodies was the event of interest while mortality was the competing risk. This method of analysis provides a more accurate estimate of the actual incidence rate than that derived from actuarial methods such as Kaplan and Meier’s as it takes death as a competing risk for RBC alloimmunization. OS was plotted using Kaplan and Meier’s survival curve and the difference between the groups was determined by log-rank tests. Alloantibodies developed following MDS-unrelated RBC transfusion and before RBC/platelet transfusion were not included when calculating cumulative incidence of RBC alloimmunization.

Factors associated with increased rate of RBC antibody formation and predicting RBC antibody formation were investigated by random survival forest (RSF), recursive partitioning (RPART) and competing risk regression (CRR) analysis. RSF technique was used to assess the importance of each variable for determining alloimmunization risk. Factors such as age at diagnosis, sex, MDS subtype, treatment, IPSS-R risk groups, number of RBC units transfused before alloimmunization, and RBC-TD or transfusion independent (RBC-TI) status were used for RSF analysis. RPART was used to create a classification tree for alloimmunization. RPART allows for the formulation of a non-biased analysis tree and highlights the importance of combinations of factors in determining risk of alloimmunization in MDS patients. RPART (v4.1-10) and RSF (v4.6-12) analyses were performed using R (R foundation for statistical computing). Lastly, competing risk regression model (CRR; with death being competing risk) was used to predict the risk of alloimmunization before and at 6-months after first RBC transfusion. For baseline analysis, age, sex, MDS subtype, IPSS-R risk groups and type of treatment were included. For the 6-month analysis, number of RBC-units transfused within 6 months and RBC-TD status at 6-months were included in addition to all the baseline factors. Categorical variables were summarised by number and frequency, and comparison between groups was done by using Fisher’s exact test or the Chi square test.
Numerical variables were summarised by mean (±SD) and comparison of numerical variables between the groups was carried out using non-parametric tests such as the Mann-Whitney or Kruskall Wallis ANOVA tests.

Supplementary Results

Case studies elucidating DHTR and autoimmune hemolysis in alloimmunized patients:

During the study period, two patients developed clinically significant hemolysis after alloimmunization.

The first patient was a 51-year-old male diagnosed with MDS-MLD with multiple comorbidities including cardiomyopathy, congestive cardiac failure, atrial fibrillation, type II diabetes mellitus, chronic obstructive pulmonary disease (COPD). He was admitted to hospital with orchitis and treated with broad-spectrum antibiotics, scrotal support and received RBC transfusions. He responded to treatment and was subsequently discharged home. However, within 5 days of discharge he represented to the emergency department with life-threatening hemolysis, acute renal failure, and cardiogenic shock necessitating prolonged ICU admission. At the time of presentation his hemoglobin was 29 g/L. LDH peaked at 1641 U/L (150-230), bilirubin peaked at 445 µmol/L, DAT was positive (Anti-IgG score 10 and anti-C3d score 10) and serological testing detected anti-K antibody (Figure S2 A-C). During the hospital admission, additional antibodies including anti-Kp<sup>a</sup> and Cw<sup>w</sup> were also detected. He responded to intravenous methylprednisolone, immunoglobulin infusion and RBC transfusions with K, Kp<sup>a</sup> and Cw<sup>w</sup> negative RBC. He recovered well from this illness and was transfused during subsequent episodes and there was no recurrence of hemolysis. He subsequently progressed to AML and was treated with two cycles of induction chemotherapy (cytarabine plus idarubicin) followed by azacitidine two cycles. Interestingly, although he continued to require regular RBC transfusion following induction chemotherapy, anti-K and Kp<sup>a</sup> were not detected after starting chemotherapy while anti-Cw<sup>w</sup> was intermittently detected. This case represents life-threatening DHTR.

The second patient was an 83-year-old man diagnosed with MDS-MLD with IPSS-R Low risk. His comorbidities included severe aortic stenosis, coronary artery disease, atrial fibrillation, interstitial pneumonitis, hypertension, depression, gastro-esophageal reflux, pancreatic cyst, glaucoma and impaired glucose tolerance test. After 6 months of RBC transfusion (2 units), he was admitted to hospital with hemoglobin level of 69 g/L without substantial change in neutrophil and platelet counts. Serology workup showed allo-anti-c, plus a pan-agglutinating autoantibody (along with free autoantibody in serum) and active hemolysis (high LDH, high bilirubin; Figure S2 D-F). There was no evidence of infection or recent change in medications. He was treated with steroid, to which he responded. Bilirubin, LDH reduced and hemoglobin stabilised and during follow up autoantibody became undetectable. As this occurred almost six months following his last RBC transfusion, transfused RBC were unlikely to be circulating at this time, hence hemolysis is most probably due to autoimmune hemolysis.

Figure legends:

**Figure S1. Overall survival (OS) and cumulative incidence of RBC-TD was significantly different in IPSS-R risk groups:** (A) Median overall survival was 74, 59, 30, 18 and 10 months in IPSS-R Very Low (n=118), Low (n=216), Intermediate (n=123), High (n=91) and Very High (n=87) risk groups respectively (p<0.001). (B) Cumulative incidence of RBC-TD was 32%, 47%, 48%, 67%, 68% in Very Low, Low, Intermediate, High and Very High risk groups respectively (p<0.001). (C) Probability of alloimmunization with death as competing
risk by number of RBC units received prior to alloantibody formation (D) DAT was positive in significantly higher number of alloimmunized patients compared to non-alloimmunized patients (84% vs. 33%; p<0.0001) (E) 80% of patients developing autoantibodies developed these either at the time of alloimmunization or within five months preceding or following alloimmunization.

Figure S2: Alloimmunization can lead to severe delayed hemolytic transfusion reaction (DHTR) and autoimmune hemolytic anemia (AIHA); (A-C) Patient MC developed severe DHTR resulting in Hb of 29 g/L, high LDH, hyperbilirubinemia and detection of Anti-K and Kpa alloantibody. Apart from transient rise in neutrophil and platelet counts during the acute episode there was no significant change in these blood counts (D-F). Patient AD developed acute drop in hemoglobin almost 6 months after last RBC transfusion. Drop in Hb was associated with detection of anti-c alloantibody, autoantibody and autoimmune hemolysis as evidenced by high LDH and bilirubin. There was no significant change in neutrophil and platelet counts during this episode.

Figure S3: Patient selection criteria used for RBC transfusion intensity analysis

Figure S4: Alloimmunization risk is significantly lower in patients receiving intensive chemotherapy and allogeneic stem cell transplant: (A) Number of RBC units transfused prior to alloimmunization was significantly higher in patients treated with disease modifying therapy compared to supportive care group (B) Patient receiving intensive chemotherapy or allogeneic stem cell transplant had significantly lower probability of alloimmunization compared to patient receiving Azacitidine (p=0.02) or best supportive care (BSC; p=0.0004).
Table S1: Clinical, alloantibody, autoantibody and transfusion characteristics of alloimmunized patients

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<th>C</th>
<th>D</th>
<th>E</th>
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<th>G</th>
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<td>Follow-up MDS diagnosis and last transfusion (months)</td>
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Columns F, G and J

Cases in which autoantibody was detected before the alloantibody, autoantibody screen was triggered by positive allo antibody screen (Ab screen positive) or was requested by treating clinicians. WAIHA-1 Diagnosed with warm autoimmune hemolytic anemia and was treated with steroids. WAIHA-2 Diagnosed with ABO-H type of warm autoimmune hemolytic anemia and was treated with steroids.
Figure S3

**Patients with alloantibodies (n=98)**
- Patients developed alloantibodies well before the MDS related transfusion (n=12)
- Patients died or progressed within four months of alloimmunization (n=15)
- Patients receiving intermittent RBC transfusions, hence unable to calculate intensity (n=11)
- Patient developed alloantibodies before documented RBC transfusion (n=7)
- Patients received only one or two units of RBC before developing alloantibodies (n=9)
- Patients were treated with Azacitidine (n=6)
- Patients did not receive or received only one RBC unit after alloantibodies (n=3)
- Patients underwent allogeneic SCT (n=2)

**Patients were eligible for RBC-intensity analysis (n=33)**
- RBC intensity analysis before and after alloantibodies
- RBC intensity analysis over 8-months period (4-months prior to and 4-months after alloimmunization)
Figure S4

A

B

Probability of alloimmunization

- Chemo & SCT
- BSC
- Azacitidine

p=0.0004

Months since first RBC transfusion

RBC units before alloimmunization

Supportive care  DMT

p<0.0001