Red cell autoimmunization and alloimmunization in myelodysplastic syndromes: prevalence, characteristic and significance

The association between red blood cell (RBC) auto- and alloantibodies has been reported, primarily in the context of warm autoimmune hemolytic anemia (AIHA). Forty to fifty percent of AIHA patients also have RBC-alloantibodies,[1,2] usually as a result of previous RBC transfusions, with autoantibodies playing very little role in their development. Conversely, the occurrence of autoantibody in alloimmunized patients is less well recognized and has primarily been reported in sickle cell disease (SCD) and thalassemia.[3-6] Importantly, autoimmunization can lead to severe AIHA[4,5,7] and make it difficult to find compatible blood. However, incidence, risk factors, and significance of RBC autoimmunization has not been reported in myelodysplastic syndrome (MDS) patients.

The aims of this analysis were to assess the incidence of RBC autoantibodies and its association with RBC alloimmunization and RBC transfusion burden in a large cohort of MDS patients enrolled in the South Australian MDS (SA-MDS) Registry. Ethical approval was obtained from all participating institutions and procedures were in accordance with the revised Declaration of Helsinki. Clinical, transfusion history, allo- and autoimmunization, and treatment details were collected until 30th June 2017. Direct antiglobulin tests (DAT), elution testing, autoantibodies, and number of RBC transfused in alloimmunized and non-alloimmunized patients were assessed (see *Online Supplementary Methods* for details).

The cumulative incidence of alloimmunization and autoimmunization was analyzed by competing-risks regression using the Fine and Gray method. Factors associated with RBC autoantibody formation were investigated by Cox proportional hazard regression analysis and all analyses were conducted in R version 3.4.4.

Nine hundred and twenty-seven patients who had been followed for at least three months were eligible for the analysis (*Online Supplementary Figure S1*). During the study period, 794 (86%) were transfused with at least one unit of RBC, including 749 patients who required RBC transfusion for management of MDS-related anemia (*Online Supplementary Methods* and *Online Supplementary Figure S1*). Clinical, demographic and treatment details of these 749 patients are summarized in *Online Supplementary Table S1*.

During follow up, 115 of 794 (14%) patients developed 203 alloantibodies (Figure 1A). Alloantibodies against Rhesus (109 of 203; 53.7%) and Kell (44 of 203; 21.6%) antigens were the most frequent, which is similar to pre-

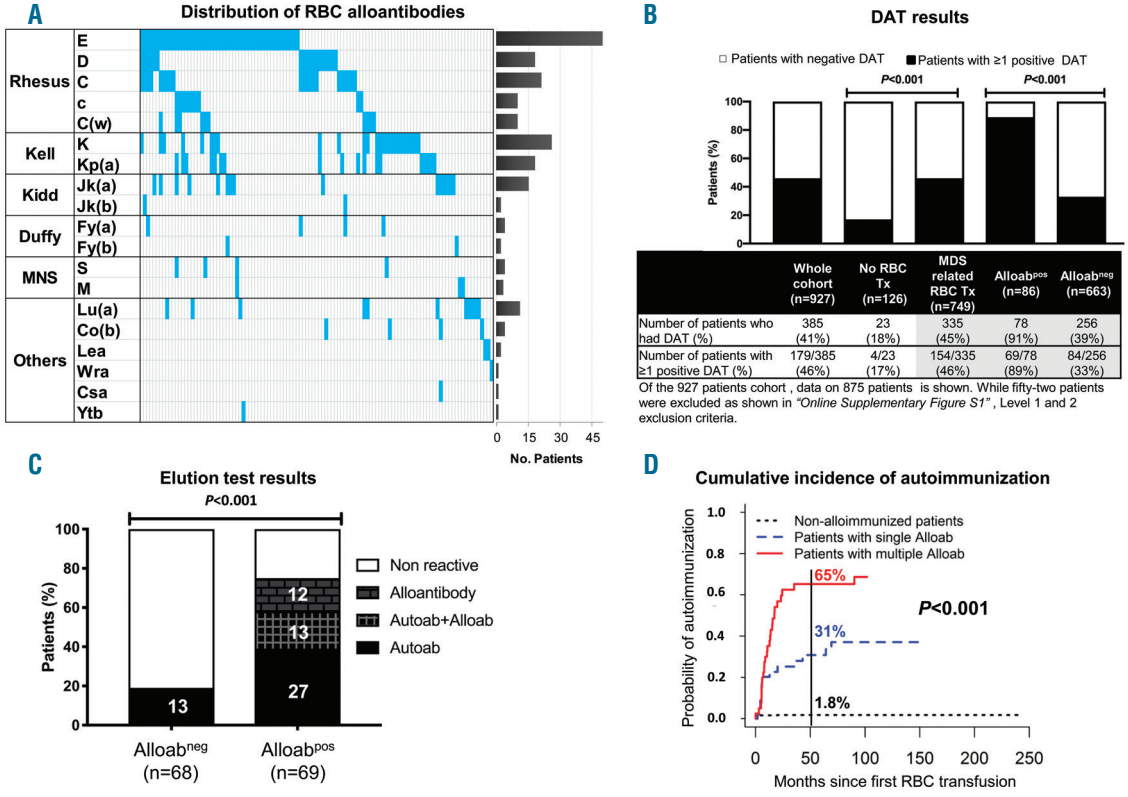

Figure 1. Distribution of alloantibodies in myelodysplastic syndromes (MDS). (A) Distribution of alloantibodies in 115 patients. Each column represents an alloantibody-positive patient and each row represents alloantibody specificity. Blue color in each box represents the presence of that specific alloantibody. The bars on the right represent the frequencies of each alloantibody in alloantibody-positive patients. (B) Comparing frequency of positive direct antiglobulin tests (DAT) in non-transfused, transfused, alloimmunized, and non-alloimmunized MDS patients. (C) Comparison of elution results between non-alloimmunized and alloimmunized patients who had elution tests. The number of patients with reactive eluates are shown in the bar diagram. (D) Cumulative incidence of autoantibody in patients with single (n=45) and multiple alloantibodies (n=41), as well as non-alloimmunized patients (n=663). No: number; RBC: red blood cell; Tx: transfusion; DAT: direct agglutination tests; Alloab: alloantibodies; Autoab: autoantibodies; pos: positive; neg: negative.

vious studies of MDS, SCD, and thalassemia patients.[3,7,8] Twenty-nine patients developed alloantibodies without documented RBC transfusions in the participating institutions, following platelets transfusions or RBC transfusions prior to MDS diagnosis for clinical indications unrelated to MDS (*Online Supplementary Figure S1*). These patients were not included in the analysis of alloimmunization following MDS-related RBC transfusion. Thus, 86 of 749 RBC transfused MDS patients developed alloantibodies with a 12.8% cumulative incidence of alloimmunization, which was comparable to the 15% reported in a study of 272 MDS patients.[8] However, studies on smaller cohorts of MDS patients reported higher alloimmunization rates, ranging from 44 to 57%.[9,10] In our study, single alloantibodies were detected in 45 of 86 (52%) alloimmunized patients, while 41 of 86 (48%) developed multiple alloantibodies.

The majority of DAT were initiated by the transfusion laboratory for further investigation of a "positive auto-control" as a part of pre-transfusion testing. A total of 1,726 DAT were performed on 385 of 927 (41%) patients and 1,206 DAT (70%) were positive. Twenty-five DAT were performed on 23 of 126 (18%) patients who did not require RBC transfusions but who had a blood group and antibody screen, and the DAT was triggered by "positive auto-control". Four of these 23 patients (17%) had at least one positive DAT (Figure 1B); interestingly, none of these four patients had autoantibodies on further testing. Of the 749 patients receiving MDS-related RBC transfusions, 335 patients (45%) had DAT, and 46% (154 of 335) of these patients had at least one positive DAT (Figure 1B). Alloimmunized patients (n=78) had much higher rates of positive DAT (89% *vs.* 33%; $P<0.001$) (Figure 1B) compared to non-alloimmunized patients (n=256) tested.

Positive DAT were further investigated by elution studies and by assessing reactivity of the eluate. Reactive eluates were significantly higher (75% *vs.* 20%; $P<0.001$) in alloimmunized (n=69) compared to non-alloimmunized (n=68) patients tested (Figure 1C). Autoantibodies, alloantibodies, combination of autoantibodies and alloantibodies, and non-reactive eluates were reported in 39%, 17%, 19%, and 25% of alloimmunized patients, respectively. Thus, 58% of eluates from alloimmunized patients tested showed autoantibody with or without alloantibody, while in non-alloimmunized patients, 80% of RBC eluates were non-reactive and only 20% of tests showed pan-agglutination due to autoantibodies (Figure 1C). The majority of the autoantibodies were non-specific except for auto-C, auto-c and auto-e, each in one patient.

Fifty-four of the 749 patients developed autoantibodies and the cumulative incidence of autoimmunization at 50 months was 6.7%, comparable to the reported incidence of 3.6-10% in MDS.[8-11] However, these studies did not compare autoimmunization in alloimmunized and non-alloimmunized patients. In our cohort, the cumulative incidence of autoantibodies was significantly higher in alloimmunized patients compared to non-alloimmunized

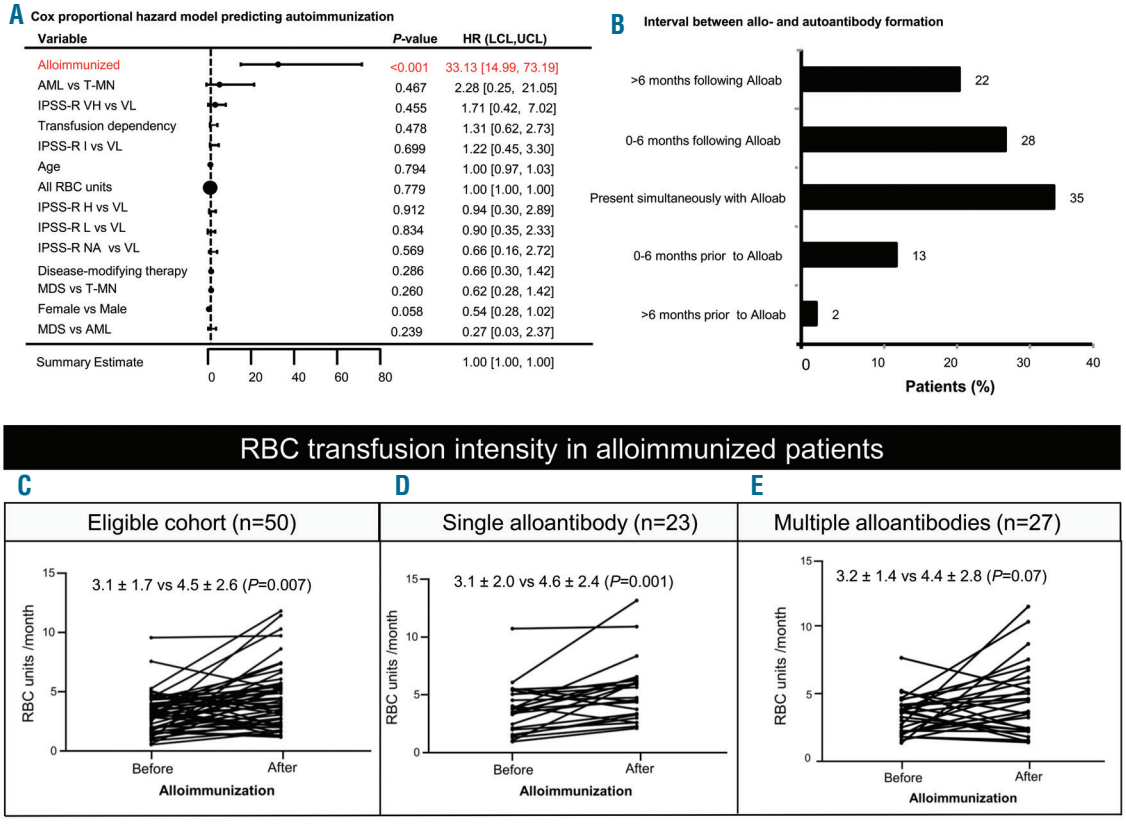

Figure 2. Alloimmunization is associated with increased risk of autoimmunization and increased red blood cell (RBC) transfusion intensity. (A) Cox proportional hazard model showing autoimmunization risk is highest in patients with alloimmunization (n=749). (B) Timing of autoimmunization in relation with alloantibodies (Alloab). RBC transfusion intensity significantly increased after alloimmunization in (C) eligible, (D) single, and (E) multiple alloantibody cohorts.

patients (47% *vs.* 1.8%; *P*<0.001). Similarly, the cumulative incidence of developing autoantibody was significantly higher in patients with multiple alloantibodies compared to a single alloantibody (65% *vs.* 31% by 50 months; *P*<0.001) (Figure 1D). Cox proportional hazard model further substantiated these findings (Figure 2A). Alloimmunization was the main risk factor for autoimmunization [Hazard Ratio (HR): 33.1; *P*<0.001]. In our cohort, 35% of autoantibodies were detected simultaneously with alloantibodies, while a further 41% of autoantibodies were detected within six months of alloantibody detection (prior to or after alloimmunization) (Figure 2B). Together, these data showed that alloimmunization is a strong risk factor for autoimmunization in MDS. A similar experience has been reported in regularly transfused SCD and thalassemia patients; autoimmunization rates range from 1-27%, with higher rates of autoimmunization in alloimmunized patients (9-69%).[3-6,12]

Autoantibody formation in alloimmunized patients can make it difficult to find compatible blood. Resolution of these complex cases translates into an increased laboratory workload and increased cost. For example, in our study, 1,117 of 1,726 (65%) DAT were performed in 103 alloimmunized patients, which constitute only 11% of the total study population. Similarly, 343 of 459 (75%) elution tests were performed in alloimmunized patients.

We and other groups have reported severe AIHA likely triggered by alloimmunization and RBC transfusion in MDS, SCD, and thalassemia patients.[4,5,7] Due to limited recognition of subclinical hemolysis in routine clinical practice and the literature, such cases likely represent only a small percentage of the true incidence. Hence, our study critically assessed the clinical implication of RBC alloimmunization and autoimmunization. The RBC transfusion intensity increased following alloimmunization in all eligible (n=50, 3.1±1.7 *vs.* 4.5±2.6; *P*=0.007), single (n=23, 3.1±2.0 *vs.* 4.6±2.4; *P*=0.001), and multiple (n=27, 3.2±1.4 *vs.* 4.4±2.7; *P*=0.07) alloantibody patients (Figure 2C-E). During the post-alloimmunized period, pre-transfusion hemoglobin (g/dL) levels were significantly lower (7.97±1.19 *vs.* 8.37±1.03; *P*<0.001), despite shorter intervals between consecutive RBC transfusions, as compared to the pre-alloimmunization period (*Online Supplementary Figure S2A and B*). Together, these data confirm and extend our previous findings[7] that RBC-alloimmunization increases RBC transfusion requirements in patients with single and multiple alloantibodies.

Presence of an autoantibody was associated with significant increase in RBC requirements in the alloimmunized patients analyzed. Autoimmunization increased RBC transfusion requirements in all eligible (n=32, 30±35 *vs.* 103±123; *P*<0.001), single (n=11, 31±36 *vs.* 109±182; *P*=0.04), and multiple (n=21, 30±36 *vs.* 100±82; *P*<0.001) alloantibody patients during the post-alloimmunization period (*Online Supplementary Figure S3A-C*). In the absence of autoantibody, RBC transfusion requirements did not increase significantly in alloimmunized patients (*Online Supplementary Figure S3D-F*). Similarly, autoimmunization increased RBC transfusion intensity in all eligible (n=32, 3.0±1.1 *vs.* 4.6±2.4; *P*=0.001), single (n=11,

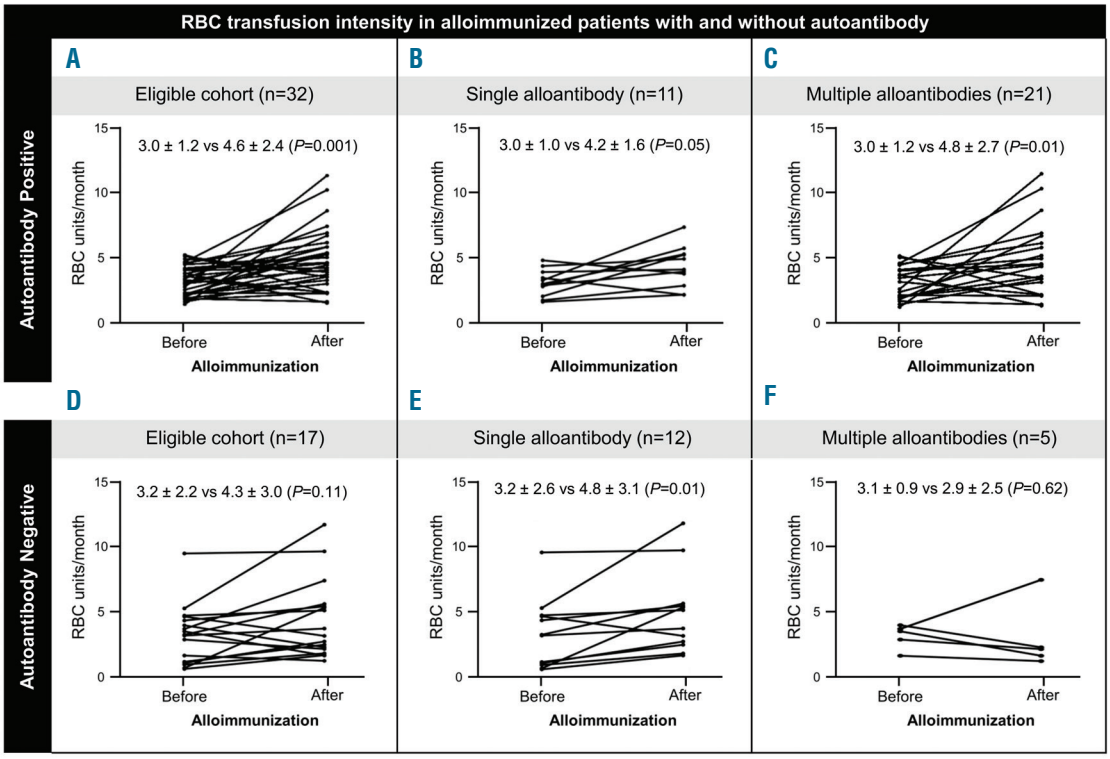

Figure 3. Autoimmunization is associated with significant increase in red blood cell (RBC) transfusion intensity in alloimmunized patients. As compared to pre-alloimmunization period, RBC transfusion intensity significantly increased during post-alloimmunization periods in (A) all eligible, (B) single, and (C) multiple alloantibody patients developing autoantibodies. While RBC transfusion requirement did not change significantly during the post-alloimmunization period in (D-F) all eligible and multiple alloantibody patients without autoantibodies, except patients with single alloantibody.

3.0 ±1.0 *vs.* 4.2±1.6; *P*=0.05), and multiple (n=21, 3.0±1.2 *vs.* 4.8±2.7; *P*=0.01) alloantibody patients following alloimmunization (Figure 3A-C). RBC transfusion intensity did not change significantly in alloimmunized patients without autoantibodies (Figure 3D-F), except in patients with single alloantibody. In alloimmunized patients with autoantibodies, pre-transfusion hemoglobin levels were significantly lower during the post-alloimmunization period, despite increased RBC transfusion frequency, as compared to pre-alloimmunization period. In autoantibody negative patients, there was no significant difference in pre-transfusion hemoglobin levels during the pre- and post-alloimmunization periods; however, transfusion frequency was higher in the post-alloimmunization period (*Online Supplementary Figure S2C*).

Despite starting with a large dataset, the number of patients with single and multiple alloantibodies with and without autoantibodies is small, which remains a limitation of the study. The applicability of these findings can be improved further by validation in larger independent cohorts.

The exact mechanism of autoimmunization following alloimmunization remains elusive. Theories include failure to regulate alloantibody-induced lymphoproliferation, altered processing and presentation of alloantigens to autologous T cells, and dampening T-regulatory cell response thus tipping the balance from regulatory towards pathogenic autoreactive T cells.[13,14]

This is the largest comprehensive study reporting alloimmunization as the most important risk factor for autoimmunization in RBC-transfused MDS patients. This study also demonstrates that increased RBC transfusion requirement following alloimmunization was mainly driven by autoimmunization, most probably due to subclinical hemolysis of autologous cells along with transfused cells. In MDS patients, assessment of hemolysis can be complicated by a higher (disease-related) baseline lactate dehydrogenase (LDH) level and poor reticulocyte response due to dyserythropoiesis. RBC transfusion can also influence LDH, haptoglobin, and bilirubin levels. Hence, a high degree of clinical suspicion is required. Strategies to decrease alloimmunization risk may be associated with decreased autoimmunization risk, which warrants further studies.

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