Isoagglutinin-reduced immunoglobulin retains efficacy in mouse models of immune thrombocytopenia and rheumatoid arthritis and is less likely to cause intravenous immunoglobulin–associated hemolysis

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BACKGROUND: Immunoglobulin therapy including intravenous immunoglobulin (IVIg) has been used as an effective treatment for autoimmune/inflammatory conditions with few side effects. However, high-dose IVIg (1-2 g/kg) has been recognized as a cause of hemolytic anemia in non–blood group O patients. Hemolysis when observed has been due to anti-A/anti-B isoagglutinins contained in the IVIg. Recently, an isoagglutinin-reduced IVIg, whereby the anti-A and anti-B titers have been reduced by immunoaffinity chromatography, has been introduced; however, whether this new product is as efficacious as nonreduced immunoglobulin (Ig) or will result in less IVIg-associated hemolysis has not been resolved.

STUDY DESIGN AND METHODS: We used in vitro phagocytosis by monocytes and proinflammatory/anti-inflammatory macrophages, with isoagglutinin-reduced and -nonreduced Ig opsonized group A_1, B, and A_1B red blood cells, to estimate clinical significance of the IgG isoagglutinins. We also used immune thrombocytopenia (ITP) and rheumatoid arthritis (RA) mouse models to examine the in vivo efficacy of isoagglutinin-reduced versus -nonreduced Ig on the amelioration of the diseases.

RESULTS: In contrast to nonreduced Ig, phagocytosis was largely absent when isoagglutinin-reduced Ig was used at a concentration equivalent to a patient receiving 2 g/kg.

The in vivo efficacy of isoagglutinin-reduced versus nonreduced Ig on the amelioration of experimental ITP and RA was similar, indicating no loss of efficacy due to the chromatographic removal of isoagglutinins.

CONCLUSION: Isoagglutinin-reduced Ig should have efficacy similar to nonreduced Ig and result in less IVIg-associated hemolysis.

Immune globulins are plasma-derived products pooled from the plasma of thousands of donors[1,2] that can be administered intravenously (IVIg) or subcutaneously. It is approved by authorities for indications such as immune thrombocytopenia (ITP), primary immunodeficiency, B-cell chronic lymphocytic leukemia, chronic inflammatory demyelinating polyneuropathy, Kawasaki disease, and multifocal motor neuropathy.[1–4]

Despite its huge success in ameliorating disease symptoms, high doses of IVIg (1-2 g/kg) have been recognized as a cause of hemolytic anemia in non–blood group O patients.[5–9] In one prospective study, approximately 35% of non–blood group O patients who received high-dose IVIg are documented to suffer from mild to severe hemolysis,[6] with group A_1B at higher risk of hemolysis than heterozygous group A_1O or group BO.[8] Other adverse events such as hypertension,

ABBREVIATIONS: FDA = US Food and Drug Administration; HSA = human serum albumin; ITP = immune thrombocytopenia; IVIg = intravenous immunoglobulin; K/BxN = mouse model that spontaneously develop rheumatoid arthritis; M1 = proinflammatory macrophages; M2 = anti-inflammatory macrophages; MMA = monocyte monolayer assay; RA = rheumatoid arthritis.

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renal failure, and thrombosis are documented, although extremely rare.

Hemolysis when observed has been due to anti-A and anti-B isoagglutinins contained in the IVIg preparations.[5–10] These isoagglutinins reacting with patients' red blood cells (RBCs) can result in hemolytic anemia through destruction of the patients' isoagglutinin-coated RBCs via the mononuclear phagocyte system through phagocytosis.[5,6,10–14] To measure the level of phagocytic activity due to antibody opsonization of RBCs, an in vitro assay, the monocyte monolayer assay (MMA), was developed.[12–14] It is an assay that is used for the prediction of clinical significance of various alloantibodies and has been used to select serologically incompatible blood for safe transfusion into patients.[13,15] Herein, the MMA is used as a measurement of clinical significance of IVIg on its potential to cause hemolysis when administered.[11]

Recently, an isoagglutinin-reduced IVIg, whereby the anti-A and anti-B titers have been greatly reduced by an immunoaffinity chromatographic approach, has been introduced into the clinic.[16] We aim to investigate whether isoagglutinin-reduced IVIg-opsonized group A_1, group B, and group A_1B RBCs results in lower phagocytosis in the MMA compared to its nonreduced immunoglobulin (Ig) counterpart. In addition, we also examined the in vivo efficacy of isoagglutinin-reduced IVIg in the amelioration of mouse models of rheumatoid arthritis (RA) and ITP.

MATERIALS AND METHODS

Immunoglobulins

Immunoglobulins used in the study were obtained from CSL Behring. Detailed information and references on the preparation of isoagglutinin-reduced Ig and non–isoagglutinin-reduced Ig is included in the Appendix S1, available as supporting information in the online version of this paper.

Monocyte monolayer assay

The MMA is a cell-based laboratory test to predict the clinical significance of a patient's antibodies against incompatible RBCs.[12–14] Detailed procedures for the MMA can be found in previous published work from our laboratory.[11,14] In brief, blood group A_1, group B, and group A_1B RBCs were opsonized with different concentrations of isoagglutinin-reduced and -nonreduced Ig (5, 10, 20, 30, and 50 mg/mL) for 1 hour, with intermittent mixing every 15 minutes. Next, 1.25% v/v opsonized RBCs in RPMI (Wisent) were added to nonactivated monocytes adhered to chamber slides and cultured for 2 hours. For the use of activated monocytes and proinflammatory (M1) and anti-inflammatory (M2) macrophages, the same assay was applied (a detailed protocol is included in Appendix S1, available as supporting information in the online version of this paper). We use phagocytic index[11] greater than 17 as the cutoff for the potential for clinical significance in the case of IVIg-associated hemolysis, as we have previously optimized the MMA to predict clinical significance of IVIg in vivo.[11]

Mouse models

Detailed procedures for the mouse ITP and RA models can be found in Appendix S1, available as supporting information in the online version of this paper. In brief, for ITP, BALB/c and C57BL/6 mice were injected daily with escalating doses of rat monoclonal anti-mouse glycoprotein IIb antibody (anti-CD41; clone MWReg30) to induce ITP.[17] For RA, we used the passive serum-transfer model (K/BxN) of RA, which has been extensively studied and has many characteristics with human RA.[18–20] Detailed procedures can be found in previous publications and Appendix S1, available as supporting information in the online version of this paper.

Statistical analysis

Statistical analysis was performed with computer software (Prism, GraphPad Software). One-way or two-way analysis of variance was applied as specified. Two tests were done including the Brown-Forsythe test and Bartlett's test. Error bars are representative of $\pm$ standard error of the mean. P values are assigned as follows: **** denotes p less than 0.0001; *** denotes p less than 0.0001 to 0.001); ** denotes p less than 0.001 to 0.01); and * denotes p less than 0.01 to 0.05). Replicates are biologic or analytical as specified.

RESULTS

An immunoaffinity chromatography step was introduced towards the end of the industrial-scale IVIg manufacturing process.[16] The final IVIg product has lower anti-A and anti-B isoagglutinins compared to a non-reduced Ig product (Table S1, available as supporting information in the online version of this paper.).

To examine whether the reduction of isoagglutinins in IVIg can significantly lower clinical hemolytic risks, we used MMA to investigate phagocytosis by monocyte–macrophages of Ig-opsonized RBCs. Figure 1A shows that the PI of human monocytes followed an increasing trend as the concentration of nonreduced Ig increased (Fig. 1A). In addition, the PI of opsonized group A_1 RBCs reached clinical significance (PI $\geq$17) at a concentration as low as 10 mg/mL (Fig. 1A). In contrast, the PI remained at a low level, considered to predict a nonhemolytic episode if infused, as the isoagglutinin-reduced Ig opsonization concentration increased; only a marginal increase in PI was observed when isoagglutinin-reduced Ig was at 50 mg/mL (Fig. 1A). To better represent clinical situations, we used an Ig opsonization concentration of 33 mg/mL, equivalent to an in vivo dose of 2 g/kg in an average-sized male of 80 kg with 4.8 L of blood (Fig. 1B). Using healthy donors' unactivated monocytes, isoagglutinin-reduced Ig significantly reduced phagocytosis compared to its nonreduced counterpart (Fig. 1B).

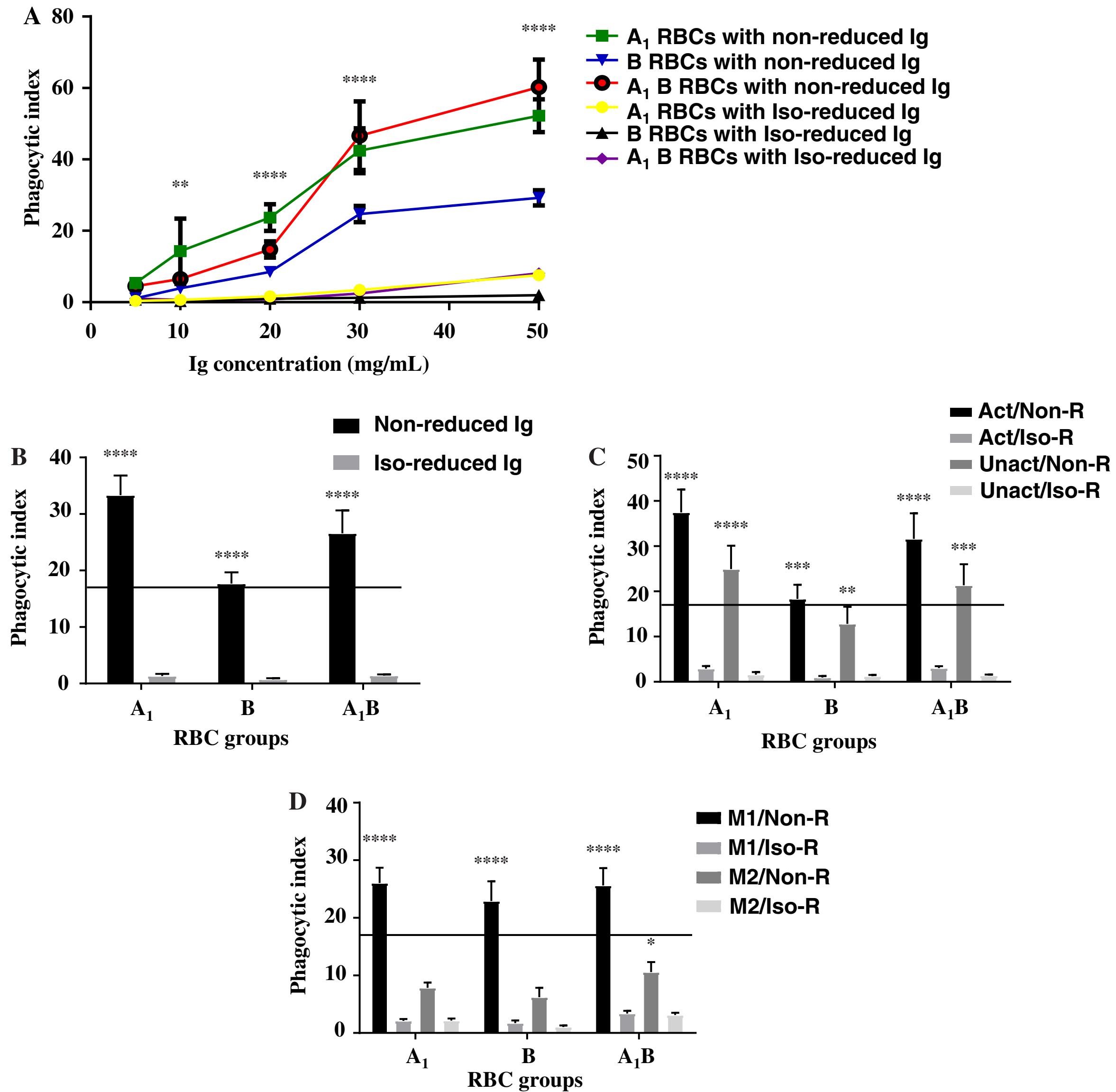

Fig. 1. MMA predicts isoagglutinin-reduced Ig is less likely than non–isoagglutinin-reduced Ig to result in in vivo hemolysis if infused into a non–group O patient. MMA was performed using healthy donors. Data represent 3 independent experiments, and two-way analysis of variance was used for statistics. (A) Group A_1, group B, and group A_1B RBCs were opsonized with isoagglutinin-reduced (Iso-reduced Ig) or -non-reduced Ig at different concentrations (5, 10, 20, 30, and 50 mg/mL; equivalent of in vivo doses of 0.3, 0.6, 1.2, 1.8, and 3 g/kg IVIg). (B) MMA with nonactivated, peripheral blood monocytes. Group A_1, group B, and group A_1B RBCs were opsonized with Iso-reduced or -non-reduced Ig at 33 mg/mL, equivalent to a dose of 2 g/kg in vivo. (C) MMA using activated (Act) or unactivated (Unact) monocytes. Group A_1, group B, and group A_1B RBCs were opsonized with iso-reduced (Iso-R) or -nonreduced (Non-R) Ig at 33 mg/mL. (D) MMA with M1 or M2 macrophages. Group A_1, group B, and group A_1B RBCs were opsonized with Iso-R or Non-R Ig at 33 mg/mL. ****$p < 0.0001$; ***$p <$ 0.0001-0.001; ** $p <$ 0.001-0.01; *$p <$ 0.01-0.05.

When RBCs were opsonized with isoagglutinin-reduced Ig, interactions were considered to be clinically insignificant (PI ≤17) (Fig. 1B).

Most patients receiving IVIg treatment suffer from inflammation associated with their disease[5]; therefore, we aimed to investigate phagocytosis with use of activated monocytes and M1 macrophages. Phagocytosis is positively correlated with activation of monocytes/macrophages.[11,21] Activated monocytes demonstrated higher phagocytosis compared to nonactivated monocytes (Fig. 1C). Both activated and nonactivated monocytes phagocytosed nonreduced Ig-opsonized RBCs effectively (Fig. 1C). However, even with activated monocytes, no clinically significant PI was observed when isoagglutinin-reduced Ig was used to opsonize RBCs (Fig. 1C). Similarly, M1 macrophages

are prominent responders to nonreduced Ig opsonized RBCs compared to M2 macrophages (Fig. 1D). However, no clinically significant PI was observed with isoagglutinin-reduced Ig (Fig. 1D).

With our in vitro phagocytosis assay, we have successfully shown that isoagglutinin-reduced Ig is predicted to significantly reduce hemolysis. However, our in vitro assay does not predict whether the chromatographic procedure to remove the isoagglutinins has affected the efficacy of the isoagglutinin-reduced Ig to ameliorate autoimmune disease. Thus, we tested and compared the efficacy of isoagglutinin-reduced Ig to nonreduced Ig with two different mouse models of autoimmune disease: ITP[17] and the K/BxN of RA.[18–20]

Injection of anti-CD41 (clone MWReg30) was able to dramatically reduce platelet number, and the escalating dose of anti-CD41 anti-platelet kept platelets at nadir numbers (Fig. 2A-B). Nontreated control had a steady platelet nadir over 3 days (Fig. 2A-B). After Ig was administered, with either

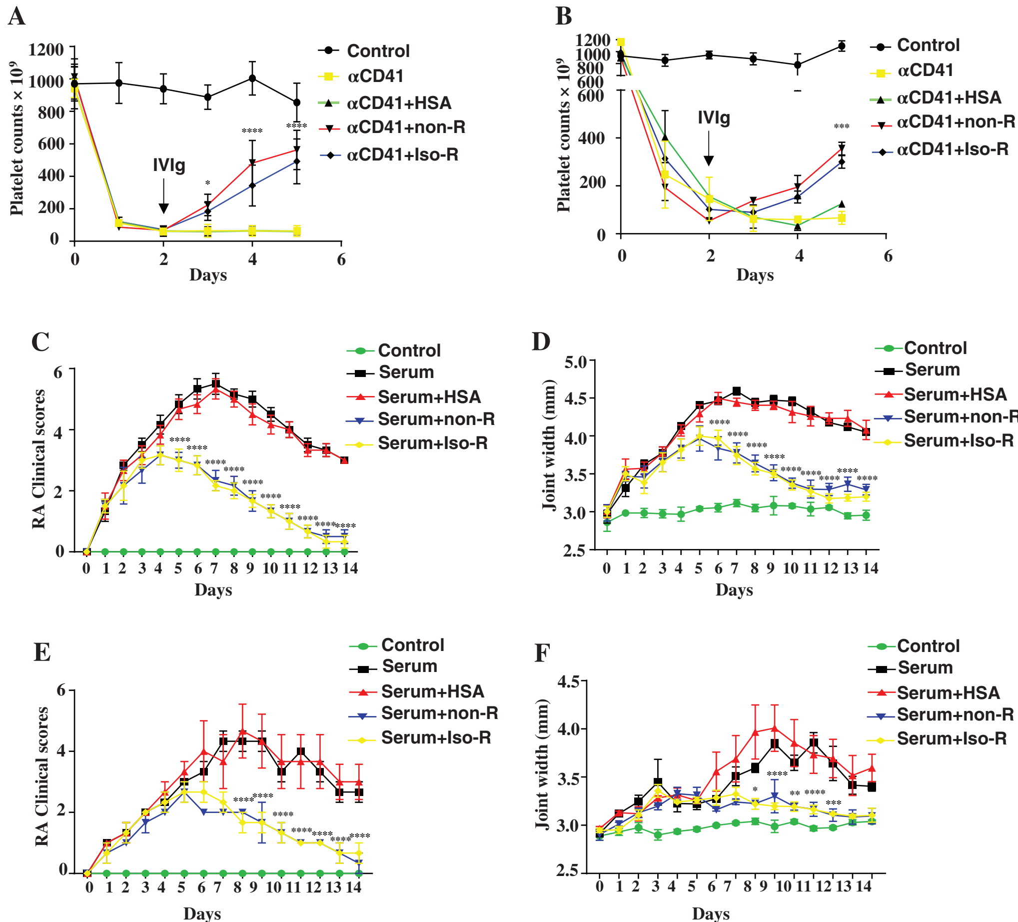

Fig. 2. The effect of Ig treatment on the amelioration of experimental ITP and RA with use of mouse models. (A) BALB/c mice were divided into five different treatment groups (n = 9 per group) including negative control, anti-CD41 (MWReg30) treatment group, anti-CD41 treatment group treated with 1 g/kg human serum albumin (HSA), anti-CD41 treatment group treated with 1 g/kg nonreduced (Non-R) Ig, and anti-CD41 treatment group treated with 1 g/kg isoagglutinin-reduced (Iso-R) Ig. (B) C57BL/6 mice were divided into five different treatment groups (n = 3 per group) as previously described 2 g/kg of HSA, non-D Ig, and Iso-D Ig was administered. (A-B) ITP was induced by injecting an escalating dose of anti-platelet (anti-CD41; clone MWReg30) every day to the mice.[17] Ig was administered on Day 2. Platelet number was assessed daily. (C-D) BALB/c mice were divided into five different treatment groups (n = 6 per group) including: no serum-transfer negative control (Control), K/BxN serum transfer group (Serum), K/BxN serum-transfer group treated with 2 g/kg HSA (Serum+HSA), K/BxN serum-transfer group treated with 2 g/kg non–isoagglutinin-reduced (Serum+non-R) Ig, and K/BxN serum-transfer group treated with 2 g/kg isoagglutinin-reduced (Iso-R) Ig. (E-F) C57BL/6 mice were divided into five different treatment groups as described above (n = 3 per group). No serum-transfer negative control (Control) and mice that received serum-transfer and treatment with 2.5 g/kg HSA (Serum), Non-R Ig, and Iso-R Ig. Arthritis clinical scores were assessed for 14 days past treatments (C and E). Joint width was also measured following the assessment of clinical scores (D and F). Two-way analysis of variance was done to calculate statistical significance. ****p < 0.0001; ***p < 0.0001-0.001; **p < 0.001-0.01; *p < 0.01-0.05).

isoagglutinin-reduced or -nonreduced Ig, platelet numbers of Balb/C (Fig. 2A) and C57BL/6 (Fig. 2B) mice gradually increased over Days 4 and 5 (Fig. 2A), with no significant differences in platelet number.

We have previously shown that non–isoagglutinin-reduced Ig is effective in treating RA in BALB/c mice with transfer of K/BxN sera.[19,20] In the work herein, injection of K/BxN serum into healthy BALB/c and C57BL/6 mice induced the development of arthritis, and clinical scores were maximal on Day 7 without any treatment (Fig. 2C and E). The slight drop in clinical scores in the control mice treated with human serum albumin past Day 7 is likely due to the depletion of the injected serum (Fig. 2C and E). Joint width measurement also corresponded with the clinical scores (Fig. 2D and F). Treatment with immunoglobulin (both isoagglutinin-reduced and -nonreduced) significantly ameliorated the disease outcomes in both mouse strains (Fig. 2C-F). Arthritis clinical scores started to drop on Day 5 and reached a clinical score close to 1 by Day 14 (Fig. 2C and E). Joint width measurement also corresponded with the clinical scores (Fig. 2Dand F). Again, isoagglutinin-reduced Ig showed the same efficacy as nonreduced Ig for the amelioration of RA.

DISCUSSION

We have shown that an isoagglutinin-reduced Ig preparation, in contrast to its nonreduced counterpart, significantly reduces phagocytosis of opsonized group A_1, group B, and group A_1B RBCs in an in vitro phagocytosis assay, below levels that would be considered to result in a clinically significant hemolytic event if infused. Using activated monocytes and M1 and M2 macrophages, we further demonstrated the nonhemolytic nature of isoagglutinin-reduced Ig if transfused into patients with acute or chronic inflammation.

IVIg is one of the US Food and Drug Administration (FDA)-approved treatments for ITP.[2,3] We confirmed, consistent with our previous publication,[17] the effect of Ig therapy on the amelioration of ITP in our mouse model. The use of isoagglutinin-reduced Ig has similar efficacy in the treatment of experimental ITP and hence should be effective for future use.

RA is a systemic autoimmune disease characterized by inflammatory arthritis and extra-articular involvement.[14,18] Although IVIg has not been FDA approved for RA treatment, our current and previous work has indicated its success in treating RA in different mouse models.[19,20] Again, isoagglutinin-reduced Ig proved to have similar efficacy in ameliorating RA disease symptoms; as a result, it has similar potential to become a treatment approach for RA with a low risk of any associated hemolysis.

Although we and others have found that an in vitro MMA can predict hemolysis in patients, and our MMA data suggest a significant reduction in hemolytic events if patients receive isoagglutinin-reduced IVIg up to 2 g/kg, a clinical trial is necessary to determine whether this outcome actually occurs. Our efficacy studies were done only in mice and then in only two mouse models of human autoimmune conditions. Indeed, while animal and in vitro models may be useful in advancing human clinical outcomes, only a human clinical trial will show the actual clinical impact.

In conclusion, in contrast to non–isoagglutinin-reduced Ig, phagocytosis observed using an MMA with isoagglutinin-reduced Ig at an equivalent in vivo dose of 2 g/kg used to opsonize RBCs, predicted a nonhemolytic event if infused into a patient of group A_1, group B, or group A_1B. Isoagglutinin-reduced and -nonreduced Igs have equal efficacy in the amelioration of ITP and RA in BALB/c and C57BL/6 mice. We predict that use of an isoagglutinin-reduced IVIg product, either by chromatographic removal or by use of a Cohn-like ethanol fractionation process,[16] would not affect the efficacy of treatment regimens but would result in fewer[8] hemolytic events in patients requiring high-dose IVIg therapy.

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CONFLICT OF INTEREST

DRB received funding and in-kind contributions from CSL Behring.

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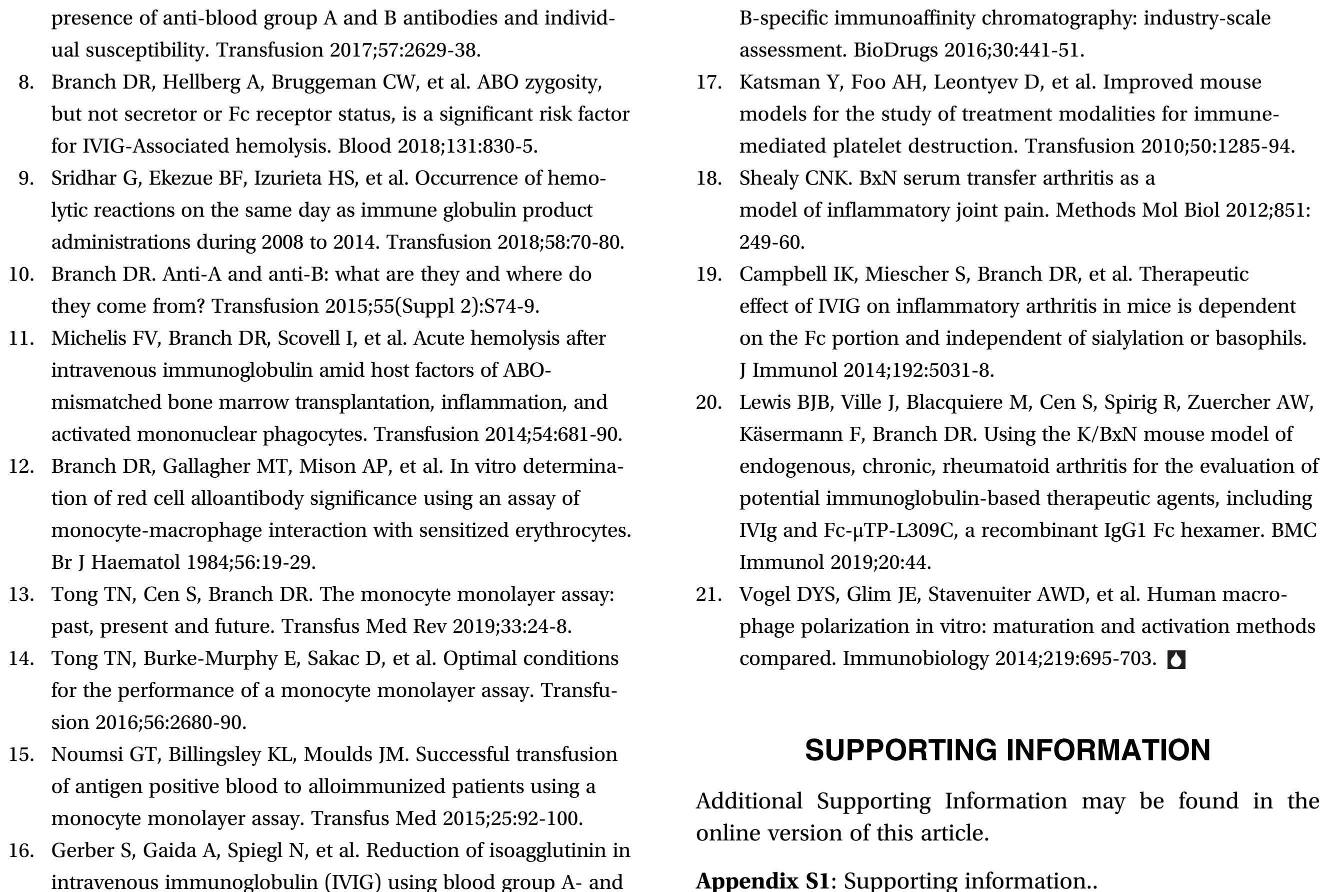

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Appendix S1: Supporting information..