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TRANSFUSION

ORIGINAL RESEARCH

Assessing and mitigating the interference of ALX148, a novel CD47 blocking agent, in pretransfusion compatibility testing

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ALX148, soluble CD47, and high-affinity SIRP monomers were provided by ALX Oncology.

Background
ALX148, a novel CD47 blocking agent, is in clinical development for the treatment of advanced solid tumors and lymphoma. Because CD47 is highly expressed on red blood cells (RBCs), its therapeutic blockade can potentially interfere with pretransfusion compatibility testing. This study describes the interference of ALX148 in pretransfusion compatibility testing and evaluates the methods used for mitigating such interference.

Study Design and Methods: Routine serologic tests were performed on six samples from four patients treated with ALX148. Antibody screening tests were performed on ALX148-spiked plasma, and RBC testing including antigen typing was performed on ALX148-coated RBCs. Soluble CD47 or high-affinity signal regulatory protein α (SIRPα) monomers were used to remove the false-positive reactivity of ALX148-spiked plasma with or without anti-E.

Results: ALX148 caused false-positive reactivity in antibody screening using indirect antiglobulin testing (IAT) and two-stage papain testing. However, false-positive reactivity was not observed at the immediate spin (IS), room temperature (RT), and 37°C phases. Direct antiglobulin testing, autologous controls, and eluates showed positive results. ALX148 did not affect blood group antigen typing performed at the IS or RT phases. The use of 50- to 100-fold molar excess of soluble CD47 or 300-fold molar excess of high-affinity SIRPα monomers removed false-positive reactivity in IAT without affecting anti-E detection.

Conclusion: ALX148 generates false-positive reactivity in IAT, interfering with pretransfusion compatibility testing. The use of soluble CD47 or high-affinity SIRPα monomers can resolve the interference without possibly missing clinically significant alloantibodies.

KEYWORDS
ALX148, CD47 blocking agents, high-affinity SIRPα monomers, Hu5F9-G4, interference, pretransfusion compatibility testing, soluble CD47

Abbreviations: IS, immediate spin; RT, room temperature; SIRPα, signal regulatory protein α; SNUH, Seoul National University Hospital.

The use of immune checkpoint inhibitors has recently emerged as one of the most promising approaches for cancer treatment.[1] Inhibitors targeting immune checkpoint molecules, such as cytotoxic T-lymphocyte–associated protein 4 and programmed cell death-1/programmed death ligand 1, have already been used in clinical practice for the treatment of a broad range of cancers.[2] The signal regulatory protein α (SIRPα)-CD47 pathway, an immune checkpoint in macrophages, is also drawing considerable attention as a potential therapeutic target.[3] As a marker of self, CD47 binds to SIRPα on the surface of macrophages to deliver an inhibitory "don't eat me" signal.[4] Cancer cells are thought to evade macrophage surveillance by up -regulating CD47 expression.[5] Several monoclonal antibodies (MoAbs) and SIRPα-Fc fusion proteins targeting CD47 are being investigated in clinical trials for the treatment of solid and hematologic cancers.[2]

CD47 is expressed on a broad range of cell types including red blood cells (RBCs).[6] Unintended binding of CD47 blocking agents to RBCs has the potential to interfere with pretransfusion compatibility testing, similar to what has been seen with CD38 blocking agents that also bind to RBCs.[7–9] Recently, the interference of a humanized anti-CD47 MoAb of the IgG4 isotype (Hu5F9-G4) in pretransfusion compatibility testing has been described.[10,11] Multiple rounds of adsorption with papain-treated allogeneic RBCs and/or indirect antiglobulin testing (IAT) using Gamma-clone anti-IgG (Immucor, Norcross, GA) without IgG4 reactivity resolved the Hu5F9-G4 interference.[10] The interference of SIRPα-Fc fusion proteins in pretransfusion compatibility testing also needs to be characterized, because their RBC-binding profiles could differ from those of anti-CD47 MoAbs.

ALX148 is a fusion protein containing a high-affinity engineered D1 domain of SIRPα genetically linked to an inactive human IgG1 Fc domain. Here, we describe the interference of ALX148 in pretransfusion compatibility testing and evaluate the various methods for mitigating such interference.

1 | MATERIALS AND METHODS

1.1 | Serologic testing of patient samples

The institutional review board of Seoul National University Hospital (SNUH) approved the retrospective chart review for this study and the in vitro experiments using ALX148-spiked plasma. Blood samples were collected as part of routine care from four patients enrolled in a phase I clinical trial of ALX148 conducted at SNUH (NCT03013218). A baseline type and screen was performed before initial ALX148 infusion. After ALX148 administration, serologic tests were conducted on patient samples as per the standard protocol of the SNUH blood bank. ABO forward grouping was performed using the tube method with anti-A and anti-B reagents (SIHDIA, Shinyang Diagnostics, Siheung, Korea). When a weak ABO subgroup was suspected, the RBCs were additionally typed using the tube method with anti-A_1 and anti-H lectins (Lorne Laboratories, Reading, UK). ABO reverse grouping was performed using the tube method with A_1 and B cells (HEMATEST, Diagast, Loos, France). The D phenotype was determined using the immediate spin (IS) tube method with two anti-D reagents (TOTEM, human monoclonal IgM/IgG blend, Diagast; SIHDIA, human monoclonal IgM/IgG blend, Shinyang Diagnostics). Antibody screening was carried out using the tube method with a two-cell panel (Selectogen I-II, Ortho-Clinical Diagnostics, Raritan, NJ) at room temperature (RT), 37°C, and albumin-IAT phases with polyspecific antihuman globulin reagents (anti-IgG and anti-C3d, Diagast). Antibody screening was also conducted using gel IAT (ID-Card LISS/antiglobulin test, BIO-RAD, Cressier, Switzerland) with a two-cell panel (ID-DiaCell I-II, BIO-RAD). Antibody identification was performed using gel IAT with an 11-cell panel (ID-DiaPanel, BIO-RAD). Direct antiglobulin testing (DAT) was performed using the gel method (ID-Card LISS/antiglobulin test). If the polyspecific DAT was positive, the patient RBCs were further tested using the gel method with monospecific anti-IgG and anti-C3d (DC-Screening II, BIO-RAD).

Samples from two patients (Samples 3-2 and 4-2) were sent to a reference laboratory (Interregional Blood Transfusion SRC Ltd., Berne, Switzerland) for further serologic testing. Polyspecific DAT was performed using the gel method (ID-Card LISS/antiglobulin test). Monospecific DAT was performed using ID-Card antiglobulin test anti-IgG (BIO-RAD) and the neutral gel card (BIO-RAD) with anti-C3d (Dako, Hamburg, Germany). Eluates were prepared using acid elution (BAG-Elutions-Kit, BAG Health Care, Lich, Germany) and tested using gel IAT (ID-Card LISS/antiglobulin test). In-house RBC panels were used for antibody screening and identification. Antibody screening was performed using the gel method (ID-Card LISS/antiglobulin test) and the tube method. The tube method was performed at the IS and low-ionic-strength solution (LISS)–IAT phases using two types of anti-IgG reagents: anti-IgG with reactivity against all human IgG subclasses (Sanquin Reagents, Amsterdam, the Netherlands) and anti-IgG without IgG4 reactivity (Gamma-clone). Additionally, 0.2 M dithiothreitol (DTT)-treated screening RBCs were used for tube LISS-IAT with Gamma-clone anti-IgG. Antibody identification was performed using gel IAT. Antibody identification was also performed using a papain-pretreated RBC panel and the neutral gel card. Antibody titration and allogeneic adsorption were carried out only in Sample 3-2. Patient plasma serially diluted with phosphate-buffered saline (PBS) at

pH 7.0 to 7.2 was tested against a pool of R_1R_1, R_2R_2, and rr RBCs using tube LISS-IAT with Gamma-clone anti-IgG. Patient plasma underwent five rounds of allogeneic adsorption using ZZAP-treated phenotype-matched RBCs (R_1r, K–). Equal volumes of ZZAP-treated RBCs, patient plasma, and LISS were mixed and incubated at 37°C for 15 minutes with periodic agitation. The mixture was centrifuged for 5 minutes, and the adsorbed plasma/LISS mixture was harvested. After four additional rounds of adsorption, the adsorbed plasma was tested using tube LISS-IAT with Sanquin anti-IgG or Gamma-clone anti-IgG. The agglutination strength was graded as 0, 0.5+, 1+, 2+, 3+, or 4+.

1.2 | Serologic testing of ALX148-spiked plasma and ALX148-coated RBCs

Discarded plasma samples (group AB with a negative antibody screen or with anti-E) were used for these experiments. Plasma with a negative antibody screen was pooled and spiked with ALX148 at final concentrations of 0.1, 1.0, 10.0, 100.0, and 500.0 μg/mL. The highest concentration (500.0 μg/mL) was expected to be above the plasma concentrations reported in patients receiving ALX148 at 10 mg/kg weekly.[12] To describe ALX148 interference in ABO, D, and extended blood group antigen typing, in vivo binding of ALX148 to RBCs was mimicked in vitro by incubating test RBCs (group O) with 500.0 μg/mL ALX148-spiked plasma (group AB) at 37°C for 15 minutes, followed by three washes with saline. The test RBCs used for this experiment were Selectogen I-II and two D– panel cells from Resolve Panel A (Ortho-Clinical Diagnostics). After incubation with ALX148, DAT was performed using the tube method with Diagast anti-IgG to evaluate whether the test RBCs were actually coated with ALX148. ABO forward typing was performed on the ALX148-coated RBCs using the tube method with SIHDIA anti-A and anti-B reagents. ABO reverse typing was performed on 500.0 μg/mL ALX148-spiked plasma using the tube method with HEMATEST A_1 and B cells. ABO forward and reverse typing was also performed using the gel method (DiaClon ABO/D + Reverse Grouping, BIO-RAD). The ALX148-coated RBCs were tested for the D phenotype using the tube method with anti-D TOTEM and SIHDIA anti-D at the IS and IAT phases. Before and after ALX148 coating, extended blood group antigen typing was conducted using the tube method with anti-C, -E, -c, -e, -K, -Jk^b, -N, and -P_1 reagents at the IS phase; anti-Le^a and -Le^b reagents at the RT phase; and anti-Fy^a, -Fy^b, -S, and -s reagents (Diagast) at the IAT phase.

To describe ALX148 interference in antibody screening, ALX148-spiked plasma was tested using gel IAT (ID-Card LISS/antiglobulin test) with ID-DiaCell I-II and the tube method with Selectogen I-II at the IS, RT, 37°C, and IAT phases. Tube IAT was performed using Diagast anti-IgG without enhancement media or with polyethylene glycol (PEG), LISS, or albumin enhancement.

1.3 | PEG adsorption

To reduce the effects of ALX148 on IAT, six rounds of PEG adsorption were performed on ALX148-spiked plasma using allogeneic R_1R_1, R_2R_2, and rr RBCs according to the method described in the *AABB Technical Manual*.[13] Briefly, equal volumes of untreated RBCs, 500.0 μg/mL ALX148-spiked plasma, and PEG were mixed and incubated at 37°C for 15 minutes with periodic agitation. After 5 minutes of centrifugation, the adsorbed plasma/PEG mixture was harvested. Five additional rounds of adsorption were carried out, and the adsorbed plasma was tested using tube IAT with Diagast anti-IgG. As a dilution control, equal volumes of 500.0 μg/mL ALX148-spiked plasma and PEG were mixed, incubated at 37°C for 15 minutes in the absence of adsorbing RBCs, and tested in the same manner.

1.4 | Mitigation using soluble CD47 or high-affinity SIRPα monomers

Mitigation studies were performed utilizing soluble human CD47 or high-affinity SIRPα monomers. Soluble CD47 and high-affinity SIRPα monomer production methods are detailed in Supplementary Methods (available as supporting information in the online version of this paper). First, we prevented the binding of ALX148 to RBCs by neutralizing the ALX148 in plasma. ALX148-spiked plasma samples were incubated with 10-, 30-, and 50-fold molar excess of soluble CD47 at RT for 30 minutes. Antibody screening using gel IAT with ID-DiaCell I-II was performed on the ALX148-spiked plasma preincubated with soluble CD47. When positive reactivity in the antibody screening remained, an additional neutralization step using 100-fold molar excess of soluble CD47 was performed. Second, we inhibited the binding of ALX148 to RBCs by masking the drug binding site on the RBCs. Screening cells (ID-DiaCell I-II) were incubated with 10-, 50-, 100-, and 300-fold molar excess of high-affinity SIRPα monomers at RT for 30 minutes under continuous shaking at 600 rpm. Antibody screening using gel IAT with the preincubated screening cells was performed on ALX148-spiked plasma. We finally demonstrated the utility of the above described mitigation strategies in ALX148-spiked plasma containing anti-E. Antibody screening using gel IAT with a three-cell panel (ID-DiaCell

I-II-III) was performed on prespiked plasma (containing anti-E only) to measure the original anti-E reactivity. Anti-E titration was not performed in the prespiked plasma. Before and after soluble CD47 (50-fold molar excess) and high-affinity SIRPα monomers (300-fold molar excess) were used, antibody screening was performed on 500.0 μg/mL ALX148-spiked plasma containing anti-E.

1.5 | Flow cytometry analysis

Flow cytometry was performed to assess ALX148 binding to test RBCs and ability of soluble CD47 or high-affinity SIRPα monomers to reduce such binding. The following three mixtures were prepared: 25 μL of ALX148-spiked plasma (0.1, 1.0, 10.0, 100.0, and 500.0 μg/mL ALX148) or normal plasma with a negative antibody screen was added to 50 μL of test RBCs (ID-DiaCell I); 25 μL of ALX148-spiked plasma (500.0 μg/mL) preincubated with 10-, 30-, and 50-fold molar excess of soluble CD47 was added to 50 μL test RBCs; and 25 μL of ALX148-spiked plasma (500.0 μg/mL) was added to 50 μL test RBCs preincubated with 10-, 50-, 100-, and 300-fold molar excess of high-affinity SIRPα monomers. The mixtures were incubated at 37°C for 15 minutes, washed four times with PBS, incubated at 4°C for 30 minutes with 0.5 μL of FITC-conjugated $F(ab')_2$ fragment goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA), and washed twice with PBS. A total of 20,000 events per sample were acquired on a flow cytometer (FACSCanto II, BD Biosciences, San Jose, CA), and RBCs were gated by forward and side scatter parameters.

2 | RESULTS

2.1 | Interference in ALX148-treated patient samples and mitigation methods

Treatment regimens and serologic test results of four patients treated with ALX148 are summarized in Table 1. ALX148 interference in ABO forward typing was evaluated in group O RBCs from Patients 2 and 4. ALX148 interference in ABO reverse typing was investigated in group AB plasma from Patients 1 and 3. In all patients, ABO forward and reverse typing gave the same results before and after ALX148 administration. All samples showed a negative antibody screen before the beginning of ALX148 treatment. After ALX148 administration, all samples showed panreactivity in both gel and tube IAT (Table 1) but nonreactive results at other phases (IS, RT, and 37°C) of the tube method. Of note, panreactivity (2+) was observed in tube IAT with Gamma-clone anti-IgG as well as with Sanquin anti-IgG (Table 1). In Samples 3-2

TABLE 1 Treatment regimens and serologic test results of four patients treated with ALX148

Patient	Sample	ALX148 regimen	ABO/D	Antibody screening		Antibody ID gel IAT	Gel DAT	Auto tube IAT	Elution gel IAT
				Gel IAT	Tube IAT				
1	1	10 mg/kg ALX148 QW with pembrolizumab	Group AB, D+	PR (3+)	PR (2+)	PR (3+)	Poly (4+), IgG (4+), C3d (0)	2+	NT
2	2	10 mg/kg ALX148 QW with rituximab	Group O, D+	PR (3+)	PR (2+)	PR (3+)	Poly (3+), IgG (3+), C3d (0)	2+	NT
3	3-1	10 mg/kg ALX148 QW with rituximab	Group AB (A_2B_3[a]), D+	PR (3+)	PR (2+)	PR (3+)	Poly (4+), IgG (4+), C3d (0)	2+	NT
	3-2[b]			PR (3+)	PR (2+)[c]	PR (3+)	Poly (4+), IgG (4+), C3d (0)	2+	PR (3+)
4	4-1	10 mg/kg ALX148 QW with pembrolizumab	Group O, D+	PR (3+)	PR (2+)	PR (3+)	Poly (4+), IgG (4+), C3d (0)	2+	NT
	4-2[b]			PR (3+)	PR (2+)[c]	PR (3+)	Poly (4+), IgG (4+), C3d (0)	2+	PR (3+)

Abbreviations: Auto, autologous control; ID, identification; mf, mixed-field agglutination; NT, not tested; Poly, polyspecific; PR, panreactive; QW, every week.

[a]The patient's RBCs showed 4+ reactivity with anti-A, $3+^{mf}$ reactivity with anti-B, no reactivity with anti-A_1, and 2+ reactivity with anti-H. The patient's plasma exhibited no reactivity with A_1 and B cells.

[b]These samples were sent to Interregional Blood Transfusion SRC Ltd. for further serologic testing.

[c]The results were same for Sanquin anti-IgG and Gamma-clone anti-IgG.

and 4-2, antibody screening using tube IAT with DTT-treated RBCs exhibited panreactivity (2+). Antibody identification using the neutral gel card with papain-treated RBCs showed panreactivity (4+). The ALX148 plasma titer was higher than 4096 in Sample 3-2. Despite five rounds of adsorption using ZZAP-treated allogeneic RBCs on plasma from Sample 3-2, 2+ reactivity remained in tube IAT with Sanquin and Gamma-clone anti-IgG.

2.2 | The effects of ALX148 on ABO, D, and extended blood group antigen typing

Test RBCs (group O) coated in vitro with ALX148 showed strong reactivity (4+) in DAT but exhibited no reactivity with anti-A and anti-B reagents in ABO forward typing using the tube and gel methods. A quantity of 500.0 μg/mL ALX148-spiked plasma (group AB) exhibited no reactivity with A_1 and B cells in ABO reverse typing using the tube and gel methods. ALX148-coated D– RBCs displayed no reactivity with anti-D reagents at the IS phase. In all extended blood group antigens tested at the IS or RT phases, antigen-negative RBCs coated with ALX148 showed no reactivity with the corresponding antigen typing reagent. When extended blood group antigen typing was performed on antigen-positive RBCs, no notable differences in agglutination strengths between pre- and post-ALX148 coating were observed. However, the 4+ DAT caused false-positive reactivity in all antigens tested at the IAT phase.

2.3 | Interference in ALX148-spiked plasma and mitigation methods

ALX148-spiked plasma exhibited panreactivity in both gel and tube IAT (Table 2). The strengths of agglutination in tube IAT were same (2+) regardless of the presence of an enhancement medium. However, agglutination was not observed in any concentration of ALX148-spiked plasma at the IS, RT, and 37°C phases. When ALX148-spiked plasma was incubated with test RBCs, the cell surface binding of ALX148 was confirmed using flow cytometry analysis (Figure 1). Binding of 0.1 μg/mL ALX148 to test RBCs was much weaker than that of ALX148 at higher concentrations (Figure 1), but it was strong enough to induce 2+ reactivity in both gel and tube IAT (Table 2). Even after six rounds of PEG adsorption using untreated allogeneic RBCs, the adsorbed plasma showed 2+ reactivity in tube IAT. The dilution control of PEG adsorption also exhibited 2+ reactivity in tube IAT. In contrast, ALX148-induced false-positive reactivity was completely mitigated using 50- to 100-fold molar excess of soluble CD47 or 300-fold molar excess of high-affinity SIRPα monomers (Table 2). The use of mitigating agents at insufficient concentrations exhibited a partial reduction of the false-positive reactivity in a gradient-dependent manner. Flow cytometry analysis revealed that the use of soluble CD47 or high-affinity SIRPα monomers reduced the binding of ALX148 to test RBCs (Figure 1). The effects of ALX148 on IAT were

TABLE 2 Mitigation of interference in ALX148-spiked plasma with or without anti-E

				Gel IAT (soluble CD47)		Gel IAT (high-affinity SIRPα monomer)	
Cells[a]	ALX148 (μg/mL)	Tube IAT	Gel IAT	50×	100×	100×	300×
I	0.1	2+	2+	0.5+	0	1+	0
II		2+	2+	0.5+	0	1+	0
I	1.0	2+	2+	0.5+	0	1+	0
II		2+	2+	0.5+	0	1+	0
I	10.0	2+	3+	0	NT	1+	0
II		2+	3+	0		1+	0
I	100.0	2+	3+	0	NT	1+	0
II		2+	3+	0		1+	0
I	500.0	2+	3+	0	NT	1+	0
II		2+	3+	0		0.5+	0
I	500.0 (with anti-E[b])	NT	3+	0	NT	NT	0
II			3+	1+			1+
III			3+	0			0

Abbreviation: NT, not tested.

[a]The Rh phenotypes of RBCs used for antibody screening tests were as follows. Screening cell I, R_1R_1; screening cell II, R_2R_2; screening cell III, R_1R_1.

[b]Anti-E in prespiked plasma showed 1+ reactivity with screening cell II (R_2R_2) in gel IAT.

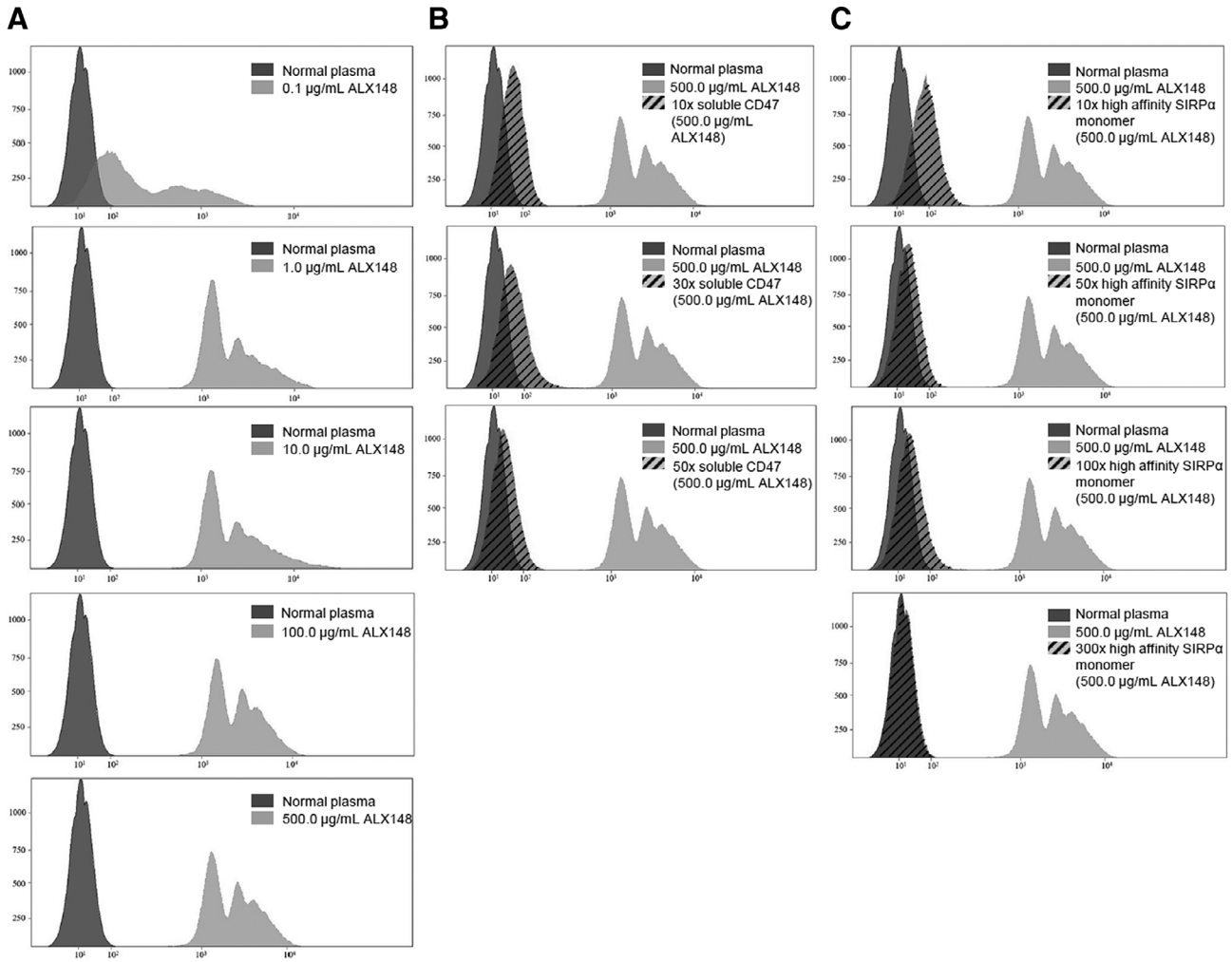

FIGURE 1 The binding of ALX148 to test RBCs and its reduction using soluble CD47 or high-affinity SIRPα monomers. In the histograms, the x-axis represents fluorescent intensity, while the y-axis represents the number of cells. A, Flow cytometry shows that ALX148 binds to test RBCs (black and gray indicate binding of normal plasma and ALX148-spiked plasma, respectively). B and C, Flow cytometry reveals that using, B, soluble CD47 or, C, high-affinity SIRPα monomers diminishes ALX148 binding to test RBCs (black, gray, and stripe indicate binding of normal plasma, ALX148-spiked plasma, and ALX148-spiked plasma after using the mitigation measure, respectively)

selectively eliminated by using soluble CD47 or high-affinity SIRPα monomers, allowing accurate identification of the underlying anti-E (Table 2).

3 | DISCUSSION

ALX148 is currently being evaluated in a clinical trial as a monotherapy or in combination with pembrolizumab, trastuzumab, or rituximab for the treatment of advanced solid tumors and lymphoma (NCT03013218).[12] However, anti-IgG used in routine serologic testing can interact with the Fc domain of ALX148 bound to the RBC membrane, thereby having the potential to induce RBC agglutination in IAT. Interference with pretransfusion compatibility testing can potentially complicate the safe and timely provision of blood products. Thus, there is an urgent need for assessing the potential of a new drug to interfere with pretransfusion compatibility testing during the early phases of drug development and introducing effective strategies to circumvent such interference.[14]

Spontaneous RBC agglutination might produce false-positive reactivity in blood group antigen typing. While RBCs from patients treated with Hu5F9-G4 showed weak spontaneous agglutination,[10] human RBCs incubated with varying concentrations of ALX148 in vitro did not agglutinate spontaneously.[15] Hence, we presumed that the impact of ALX148 on blood group antigen typing performed at the IS or RT phases would be minimal, if any at all. Indeed, our study involving samples from four patients treated with ALX148 as well as artificial samples (ALX148-coated RBCs and ALX148-spiked plasma

prepared in vitro) demonstrates that ALX148 does not interfere with blood group antigen typing performed at the IS or RT phases. To provide extended antigen-matched RBC units, it would be prudent to obtain a baseline ABO, D, and extended blood group antigen profile before initiating ALX148 therapy. After ALX148 therapy has begun, along with genotyping, phenotyping with antigen typing reagents used at the IS or RT phases can also be performed as long as the patient has not been recently received a transfusion.

As seen with CD38 blocking agents, including daratumumab,[7–9] CD47 blocking agents generate panreactivity in IAT, interfering with pretransfusion compatibility testing.[10,11,16,17] While plasma from patients treated with daratumumab typically shows weak reactivity in IAT,[18] plasma from patients treated with Hu5F9-G4 or ALX148 exhibits strong reactivity in IAT.[10,16,17] Similarly, in our study, strong reactivity (3+) in gel IAT was observed in the plasma from all four patients treated with ALX148. Retesting patient samples that initially exhibit daratumumab interference in gel IAT or solid-phase testing with the tube method can partially obviate the need for routine DTT treatment, because the tube method may not be able to detect the effects of daratumumab on IAT in some cases.[19] However, our study and that of Velliquette et al.[10] reveal that the panreactivity in IAT caused by ALX148 or Hu5F9-G4 cannot be prevented using the tube method. Experiments using ALX148-spiked plasma suggest that even very low concentrations of ALX148 in patient plasma can bind to test RBCs strongly, generating 2+ reactivity in both gel and tube IAT. Thus, panreactivity in IAT might persist for a period of time after the last ALX148 infusion. The greater reactivity of CD47 blocking agents compared to that of CD38 blocking agents is probably due to the fact that CD47 is expressed at higher levels than CD38 in the RBC membrane.[10] It should be noted that the reactivity of CD47 blocking agents may vary with the Rh phenotype of RBCs used for pretransfusion compatibility testing, because CD47 expression levels differ among different Rh phenotypes.[10] In this study, when antibody screening and identification tests were performed on plasma samples from patients treated with ALX148, the reactivity with all reagent RBCs, including rr RBCs which have the highest CD47 expression, was consistent in strength and phase regardless of their Rh phenotypes. Velliquette et al. reported that the use of D– – and Rh_{null} RBCs with markedly decreased CD47 expression failed to significantly reduce false-positive reactivity induced by ALX148.[16] The potential influence of Rh phenotypes on the mitigation strategies used in this study (soluble CD47 and high-affinity SIRPα monomers) requires further investigation as we utilized only R_1R_1 and R_2R_2 cells.

Patterns of interference in pretransfusion compatibility testing can differ among CD47 blocking agents depending on their drug classes, IgG subclasses, and RBC-binding capacity. For example, Hu5F9-G4 interferes with ABO typing and all phases of antibody screening, including IS.[10] However, as seen with daratumumab,[18] ALX148 interferes with antibody screening at the IAT phase but does not affect other phases of antibody screening as well as ABO typing. The comparison of interference in pretransfusion compatibility testing and mitigation strategies between Hu5F9-G4 and ALX148 is shown in Table 3. Although denaturing CD47 on test RBCs may be a universal approach to overcome the interference of all CD47 blocking agents, CD47 is not denatured by DTT or proteolytic enzymes such as papain.[10] Our study demonstrated that ALX148 reacted with 0.2 M DTT-treated RBCs in tube IAT and two-stage papain-treated RBCs in the neutral gel card. Velliquette et al.[16] also observed that ALX148 reacts with two-stage papain-treated RBCs at the IAT phase. Alternatively, adsorption techniques can be viable options to negate the interference of CD47 blocking agents owing to the high expression of CD47 on adsorbing RBCs. Multiple rounds of adsorption using papain-treated allogeneic RBCs (R_1R_1, R_2R_2, and rr) successfully eradicated panreactivity of patient plasma induced by Hu5F9-G4.[10] Our study showed that five rounds of adsorption using ZZAP-treated allogeneic RBCs (R_1r) failed to remove plasma panreactivity in a patient treated with ALX148, and six rounds of PEG adsorption using untreated allogeneic RBCs (R_1R_1, R_2R_2, and rr) failed to eliminate the panreactivity of 500.0 μg/mL ALX148-spiked plasma. However, Velliquette et al.[16] reported that plasma panreactivity was eliminated by six rounds of adsorption using papain-treated allogeneic RBCs (rr) in a patient treated with ALX148, and the elimination might have been attributed to the short incubation time (10 minutes). Although allogeneic adsorption may be effective in eliminating ALX148-induced panreactivity, it is laborious and time-consuming for routine clinical use and carries the risk of adsorbing alloantibodies against high-frequency antigens. The use of anti-IgG without IgG4 reactivity, such as Gamma-clone anti-IgG, proves effective in nullifying the effects of IgG4-based CD47 blocking agents on IAT.[10] In contrast, this mitigation method does not work against IgG1-based CD47 blocking agents, including ALX148.

The interference of CD47 blocking agents in pretransfusion compatibility testing can be avoided by neutralizing the drug in plasma or masking the drug binding site on RBCs. We demonstrated that the binding of ALX148 to test RBCs could be selectively inhibited using an excess of soluble CD47 or high-affinity SIRPα monomers. A disadvantage of these mitigation methods

TABLE 3 Comparison of interference in pretransfusion compatibility testing and mitigation strategies between Hu5F9-G4[10,11] and ALX148 (our study)[15-17]

	Hu5F9-G4	ALX148
Drug type	MoAb	Fusion protein
IgG subclass	IgG4	Engineered IgG1
Spontaneous agglutination	Observed	Not observed
Interference		
ABO typing	Observed	Not observed
D and extended blood group antigen typing	Possible at IS and IAT phase	Observed at IAT phase
Antibody screening and identification	Observed at all phases	Observed at IAT phase[a]
DAT/autologous control	Negative or weakly positive	Positive
Eluate IAT	Positive	Positive
Mitigation strategies		
DTT or enzyme treatment	Not effective	Not effective
IAT using anti-IgG without IgG4 reactivity	Effective	Not effective
Multiple rounds of allogeneic adsorption	Effective	Variable[b]
Soluble CD47	Probably effective (not tested)	Effective
High-affinity SIRPα monomers	Probably effective (not tested)	Effective

[a]Exceptionally, plasma strongly reacted with papain-treated RBCs in the neutral gel card.
[b]The effectiveness of allogeneic adsorption can vary depending on many factors, including the number of adsorption procedures, enzyme treatment and Rh phenotype of adsorbing RBCs, plasma concentration of ALX148, enhancement medium, and incubation time.

is that large volumes of soluble CD47 or high-affinity SIRPα monomers are required to mitigate the interference induced by high concentrations of ALX148. In this study, when 50-fold molar excess of soluble CD47 or 300-fold molar excess of high-affinity SIRPα monomers were used to eradicate the false-positive reactivity of 500.0 μg/mL ALX148-spiked plasma, the total incubation volume in gel IAT increased by almost 50% (soluble CD47) or 200% (high-affinity SIRPα monomers). Although such increases did not affect anti-E detection in this study, it is important to understand that adding soluble CD47 or high-affinity SIRPα monomers carries the risk of diluting out very weakly reactive clinically significant alloantibodies. One peculiar finding was that the minimum molar excess of soluble CD47 needed for neutralization was different at low and high concentrations of ALX148 (100-fold vs 50-fold). Such different neutralization efficiencies may be explained by the collision theory, stating that as reactant concentration increases, the number of collisions between reactant molecules increases, leading to a faster rate of reaction. The use of soluble CD47 or high-affinity SIRPα monomers could be a universal solution to the interference caused by any CD47 blocking agent, although both methods need to be validated for each CD47 blocking agent. Another alternative is the use of pepsin- or papain-digested CD47 blocking agents lacking the Fc domain. This approach has only been validated for daratumumab[20,21] but could potentially work for CD47 blocking agents. A panel of antigen typed RBCs with decreased CD47 expression may be useful to minimize the effects of CD47 blocking agents. Unlike daratumumab interference, which can be mitigated using a panel of antigen-typed cord RBCs with extremely low CD38 expression,[22] the interference induced by Hu5F9-G4 or ALX148 cannot be resolved using RBCs with significantly decreased CD47 expression, such as D– – and Rh_{null} RBCs.[10,16] Finally, the provision of phenotypically or genotypically matched RBC units is a general solution to address drug interference in pretransfusion compatibility testing. The major limitations are that extended phenotype or genotype matching is not available in many countries, and even if available, clinically significant antibodies might be missed, depending on the degree of matching.[9]

In summary, we demonstrated that ALX148 binds to test RBCs, thereby interfering with the IAT phase of pretransfusion compatibility testing, although it does not confound tests performed at other phases, including IS and RT. Various methods were investigated to mitigate ALX148-induced interference. The use of soluble CD47 or high-affinity SIRPα monomers proves effective in resolving the interference without possibly missing clinically significant alloantibodies. Phenotyping of patient RBCs for extended antigen-matched blood transfusion can be accomplished using antigen typing reagents at the IS or RT phases, even after ALX148 administration. The increasingly widespread

use of MoAbs and fusion proteins with unintended binding to RBCs poses a growing challenge to blood banks. International efforts should be undertaken to investigate the potential of each drug to interfere with pretransfusion compatibility testing and establish a guideline to solve the problem.

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CONFLICT OF INTEREST

Janet Sim and Hong I. Wan are employed by ALX Oncology. The other authors declare no potential conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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