Human Immunology 80 (2019) 468–477

Contents lists available at ScienceDirect

Human Immunology

journal homepage: www.elsevier.com/locate/humimm

Getting on target: Development of the novel, prozone-resistant, dual antibody rapid test (DART) for the LABScreen single antigen bead (SAB) assay

Anna Greenshields[a], Robert A. Bray[b], Howard M. Gebel[b], Robert S. Liwski[a,*]

[a] Department of Pathology, Dalhousie University, Halifax, Nova Scotia B3H 1V8, Canada
[b] Department of Pathology, Emory University Hospital, Atlanta, GA 30322, USA

ARTICLE INFO

Keywords:
HLA antibodies
Single antigen bead assay
Assay optimization
Transplantation

ABSTRACT

A major limitation of the single antigen bead (SAB) assay is the so called prozone effect, whereby the detection of high titer complement fixing HLA antibodies is compromised due to complement split product (from C3 and C4 components) deposition and interference with the reporter anti-IgG-PE antibody binding. Strategies to minimize prozone include serum titration or treatment with heat, dithiotreitol (DTT), or ethylenediaminetetraacetic acid (EDTA). While effective, these treatments may compromise HLA antibody binding and detection. Here we describe the **D**ual **A**ntibody **R**apid **T**est (**DART**), a modified version of the **r**apid **o**ptimized SA**B** (**ROB**) protocol, in which we use an IgG-PE/C3d-PE antibody cocktail to simultaneously detect bead bound IgG and C3d, which allows for detection of HLA antibodies independent of the prozone effect. Twenty prozone positive sera (10 class I and 10 class II), identified by titration, were tested by the ROB protocol, with or without EDTA pre-treatment, using three reporter antibody cocktails: (1) IgG-PE, (2) C3d-PE, or (3) IgG-PE/C3d-PE (DART). Mean fluorescence intensity (MFI) values were then compared. IgG negative (n = 735) vs IgG positive (n = 1185) reactions were identified using a 1000 MFI IgG EDTA cutoff. IgG positive reactions were classified based on ΔMFI (IgG EDTA – IgG) as follows: (1) prozone negative (ΔMFI < 3000; n = 737), (2) slight prozone (ΔMFI 3001–5000; n = 49), (3) moderate prozone (ΔMFI 5001–10,000; n = 93), and (4) marked prozone (ΔMFI > 10,001; n = 306). No C3d deposition was present on IgG negative beads, and the majority of prozone positive specificities (438/448; 98%) fixed complement and were detected with the C3d-PE reporter. Interestingly, C3d-PE MFI was directly proportional to the degree of prozone (mean C3d-PE MFI = 4419.5 ± 1606.3 for slight, 5991.0 ± 2302.7 for moderate, and 12,417.4 ± 2969.9 for marked prozone specificities). Interestingly, EDTA treatment was found to have a negative impact on MFI of up to 15% of prozone negative specificities. Importantly, the DART protocol detected all prozone positive specificities while MFI for prozone negative specificities correlated well with those seen with the IgG-PE reporter alone (R^2 = 0.97). In conclusion, the DART protocol accurately detects HLA antibodies independent of the prozone effect. Implementation of DART is an easy way to overcome the prozone effect without compromising HLA antibody detection.

1. Introduction

The single antigen bead (SAB) luminex assay is a fluorescence-based test, which uses polystyrene microparticles (or beads) each coated with an individual human leukocyte antigen (HLA) to specifically detect and identify HLA antibodies in recipient sera [1,2]. In the last two decades the SAB assay has emerged as a cornerstone of histocompatibility testing [3,4] and is now commonly used in virtual crossmatching [5–9] and in the assessment of immunological risk both pre- [10,11] and post-transplantation [12,13]. However, the SAB assay suffers from several limitations, which may affect test interpretation and clinical outcomes [14–17].

Perhaps the most notable of these limitations is the prozone-like effect [18,19]. In the traditional sense, the prozone effect refers to an artefact that can occur with several immunoassays, whereby the presence of excess analyte (leading to a high antigen to antibody ratio)

Abbreviations: DTT, dithiotreitol; EDTA, ethylenediaminetetraacetic acid; MFI, mean fluorescence intensity; PE, phycoerythrin; ROB, rapid optimized SAB assay; SAB, single antigen bead; DART, dual antibody rapid test; LWB, LABScreen wash buffer

* Corresponding author at: Dr. D.J. Mackenzie Building, HLA Typing Laboratory, 5788 University Avenue, Room 625, Halifax, Nova Scotia B3H 1V8, Canada.
E-mail address: robert.liwski@nshealth.ca (R.S. Liwski).

https://doi.org/10.1016/j.humimm.2019.04.017
Received 3 March 2019; Received in revised form 20 April 2019; Accepted 25 April 2019
Available online 28 April 2019

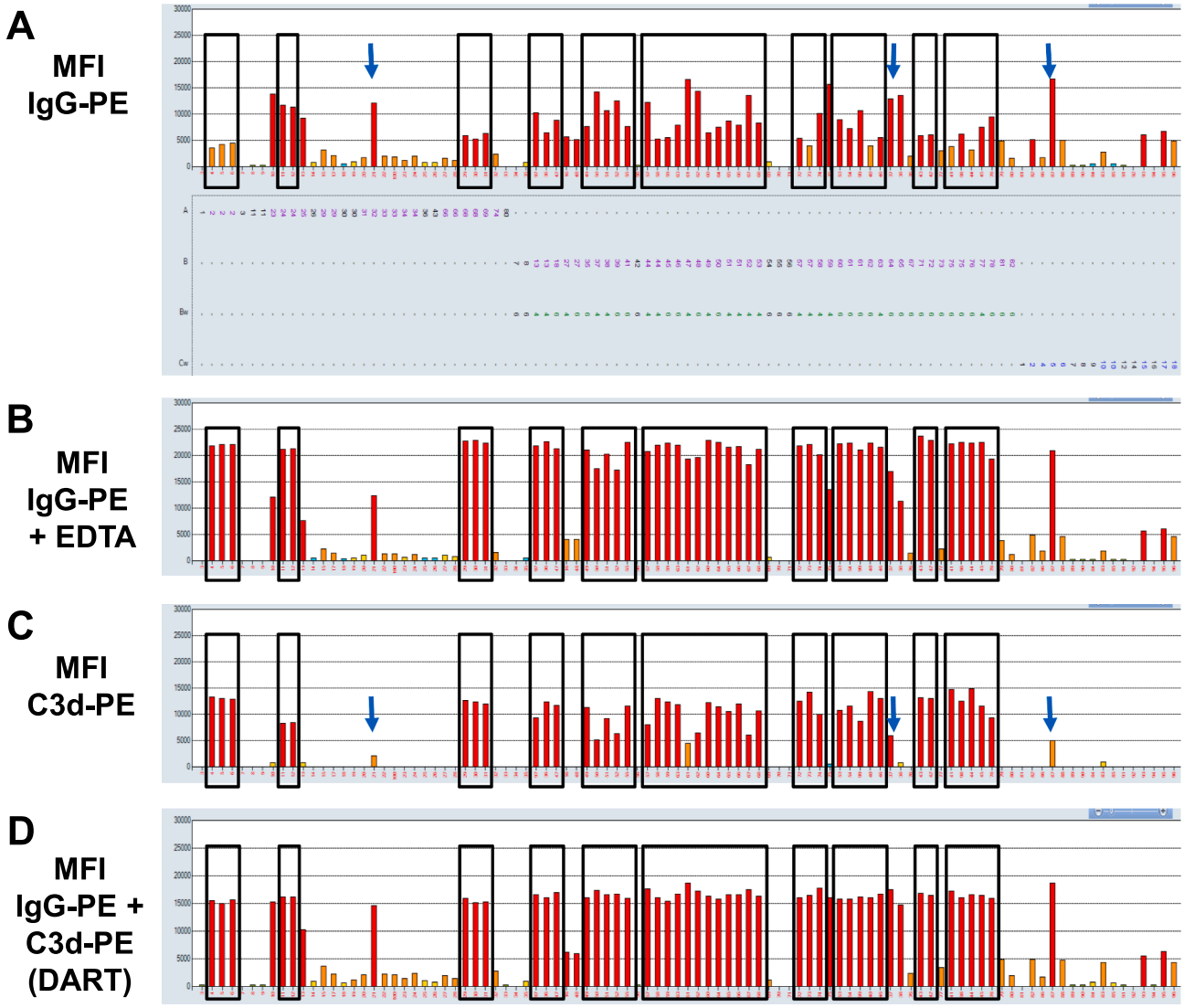

Fig. 1. Class I HLA Prozone positive specificities are detected using the C3d-PE or the combination of IgG-PE and C3d-PE reporter antibodies (DART protocol). Representative SAB images of Class I HLA specificities identified in one patient sera using the IgG-PE ± EDTA (panels A and B), C3d-PE (panel C), and the DART (panel D) protocols are shown. Specificities highlighted in black boxes are affected by prozone (ΔMFI ≥ 5000; ΔMFI = IgG-PE + EDTA MFI – IgG-PE MFI). Specificities outside the boxes are either IgG-PE negative or are not affected by prozone. Blue arrows (panels A and C) indicate prozone negative specificities that stain positively with C3d-PE (C3d-PE MFI > 1000).

causes a falsely low or negative reaction, which becomes stronger as the sample (analyte) is diluted [20]. In contrast, in the SAB assay the effect appears to be due to interference with the binding of the anti-IgG-PE secondary antibody [21,22]. Specifically, the interference occurs as a result of complement activation by high titer HLA alloantibodies and subsequent binding of complement split products (C3 and C4) to the alloantibody/antigen complexes on the SABs, which in turn inhibits the binding of anti-IgG-PE to the target alloantibody [23,24]. Our recent study, which demonstrates that decreasing the incubation temperature to 4 °C in the SAB assay (reducing complement activation and deposition) completely eliminates the prozone effect, is consistent with the notion that the interference is complement dependent [25,26].

High titer, complement fixing donor specific HLA alloantibodies pose a significant immunologic risk in transplantation, therefore, interference with detection of such antibodies in the SAB assay may have serious clinical implications including antibody mediated rejection and graft loss. Current strategies employed to prevent complement-mediated interference in the SAB assay include serum dilution [27], treatment with heat (56 °C for 30 min) [28], dithiothreitol (DTT) [18,19], or calcium chelation using ethylenediaminetetraacetic acid (EDTA) [21,22,29], which either dilute out/eliminate complement factors or inhibit complement activation. Although these techniques are effective in minimizing the prozone effect, there is some concern that that the treatment of sera could affect antibody binding and/or detection. In this manuscript we describe a novel modification of the SAB assay that minimizes the prozone effect without the need for the pre-treatment of sera. This protocol, called the dual antibody rapid test (DART), is based on our previously developed rapid optimized SAB (ROB) method [30] and uses a combination of anti-IgG-PE and anti-C3d-PE secondary antibodies to ensure the detection of HLA alloantibodies independent of complement mediated interference.

2. Materials and methods

2.1. Reagents and sera

The SAB assay was performed using LABScreen SAB kits (LS1A04 lot 10 and LS2A01 lot 11 for HLA Class I and Class II, respectively; One Lambda, Canoga Park, CA). LABScreen wash buffer (LWB), phycoerytherin (PE)-conjugated goat anti-human IgG (IgG-PE) polyclonal

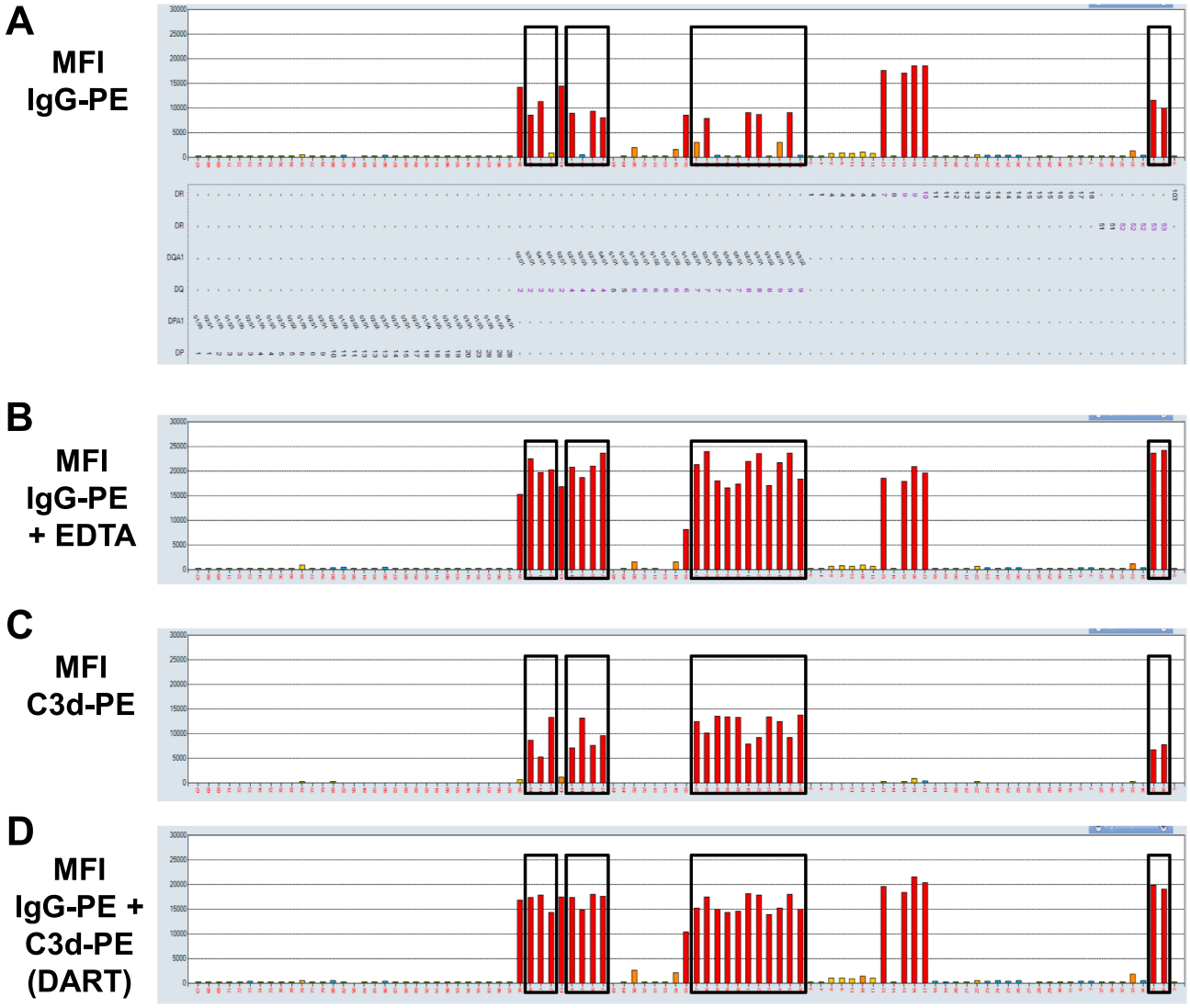

Fig. 2. Class II HLA prozone positive specificities are detected using the C3d-PE or the combination of IgG-PE and C3d-PE reporter antibodies (DART protocol). Representative SAB images of Class II HLA specificities identified in one patient sera using the IgG-PE ± EDTA (panels A and B), C3d-PE (panel C), and the DART (panel D) protocols are shown. Specificities highlighted in black boxes are affected by prozone (ΔMFI ≥ 5000; ΔMFI = IgG-PE + EDTA MFI − IgG-PE MFI). Specificities outside the boxes are either IgG-PE negative or are not affected by prozone.

antibody and PE-conjugated donkey anti-human IgM (IgM-PE) were also purchased from One Lambda. PE-conjugated anti-C3d (C3d-PE) polyclonal antibody was purchased from Immucor (Norcross, GA). 96-well v-bottom trays were purchased from Whatman (Pistcataway, NJ). EDTA disodium 0.5 M salt solution (E7889; Sigma-Aldrich, St Louis, MO www.sigmaaldrich.com) was used to treat sera in some experiments by adding 1 µl of 165 mM working EDTA solution per 25 µl of test sera [19].

Twenty prozone positive sera (10 class I HLA and 10 class II HLA) from highly sensitized (cPRA > 95%) waitlist patients were selected based on previous serum titration and EDTA protocol validation studies.

2.2. Modified SAB assay protocols

The ROB assay protocol was performed as described previously [30]. In brief, EDTA-treated or untreated sera (25 µl/test) were added to the wells of a 96-well v-bottom tray containing either HLA Class I or Class II SABs. The trays were then incubated for 15 min at room temperature in the dark on a tray shaker. Beads were washed 3 times with LABScreen wash buffer (LWB) (200 µl/well) for 1 min at 1800 × g. Following washing, 20 µl of one of three reporter antibody cocktails was added to each well. Reporter cocktails were as follows: 1) IgG-PE (1:10 dilution in LWB), 2) C3d-PE (1.5:10 dilution in LWB), and 3) IgG-PE + C3d-PE combination (1:10 and 1.5:10 dilution in LWB, respectively), named the dual antibody rapid test (DART). In some experiments IgM-PE reporter antibodies were used. Trays were incubated for 5 min at room temperature, and then washed twice with LWB as described above. Beads were then resuspended in 55 µl of LWB and were acquired using the Luminex FlexMAP 3D™ analyzer (Luminex). The raw trimmed mean fluorescence intensity (MFI) determined for each reactivity was analyzed using Fusion 3.0 software (OneLambda) and Microsoft Office Excel (Microsoft) and compared between the different treatment conditions. Positive control and negative control bead MFI values were also analyzed and compared between conditions.

2.3. Statistical analysis

Pearson's correlation coefficient was performed using Microsoft Office Excel software. Repeated measure ANOVA with the Bonferroni multiple comparison post-test was performed using GraphPad InStat (GraphPad Software Inc., San Diego, CA)

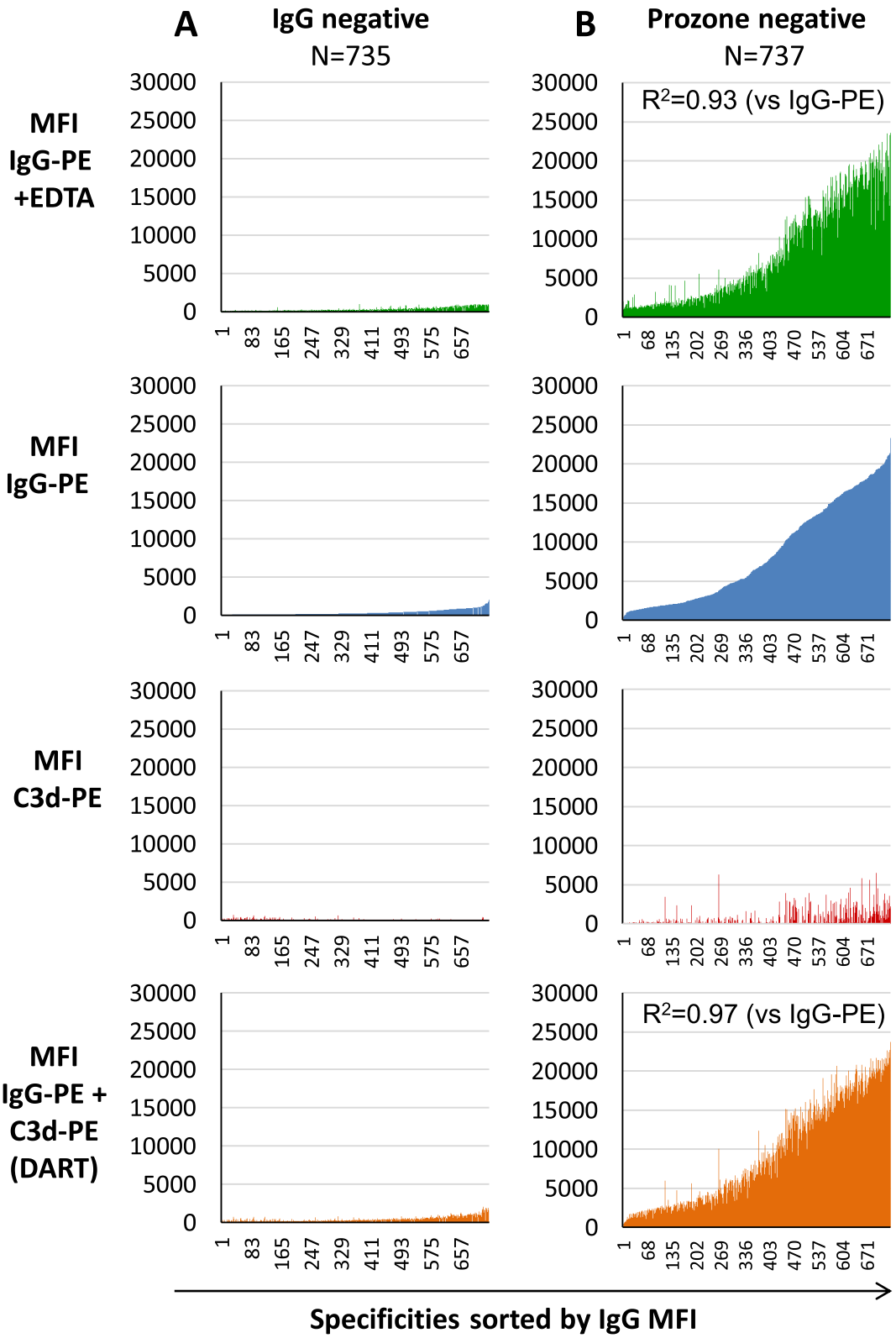

Fig. 3. Reactivity patterns of IgG negative and prozone negative specificities. SAB reactivity of 20 prozone positive patient sera tested with the IgG-PE ± EDTA, C3d-PE, and the DART protocols. Data shown compares IgG-PE negative (panel A; IgG = PE MFI < 1000) and prozone negative (panel B; ΔMFI < 3000) specificities. ΔMFI = IgG-PE + EDTA MFI – IgG-PE MFI. R^2 values for IgG-PE vs IgG-PE + EDTA MFI and IgG-PE vs DART MFI for prozone negative specificities are indicated (panel B). All specificities are sorted by IgG-PE MFI.

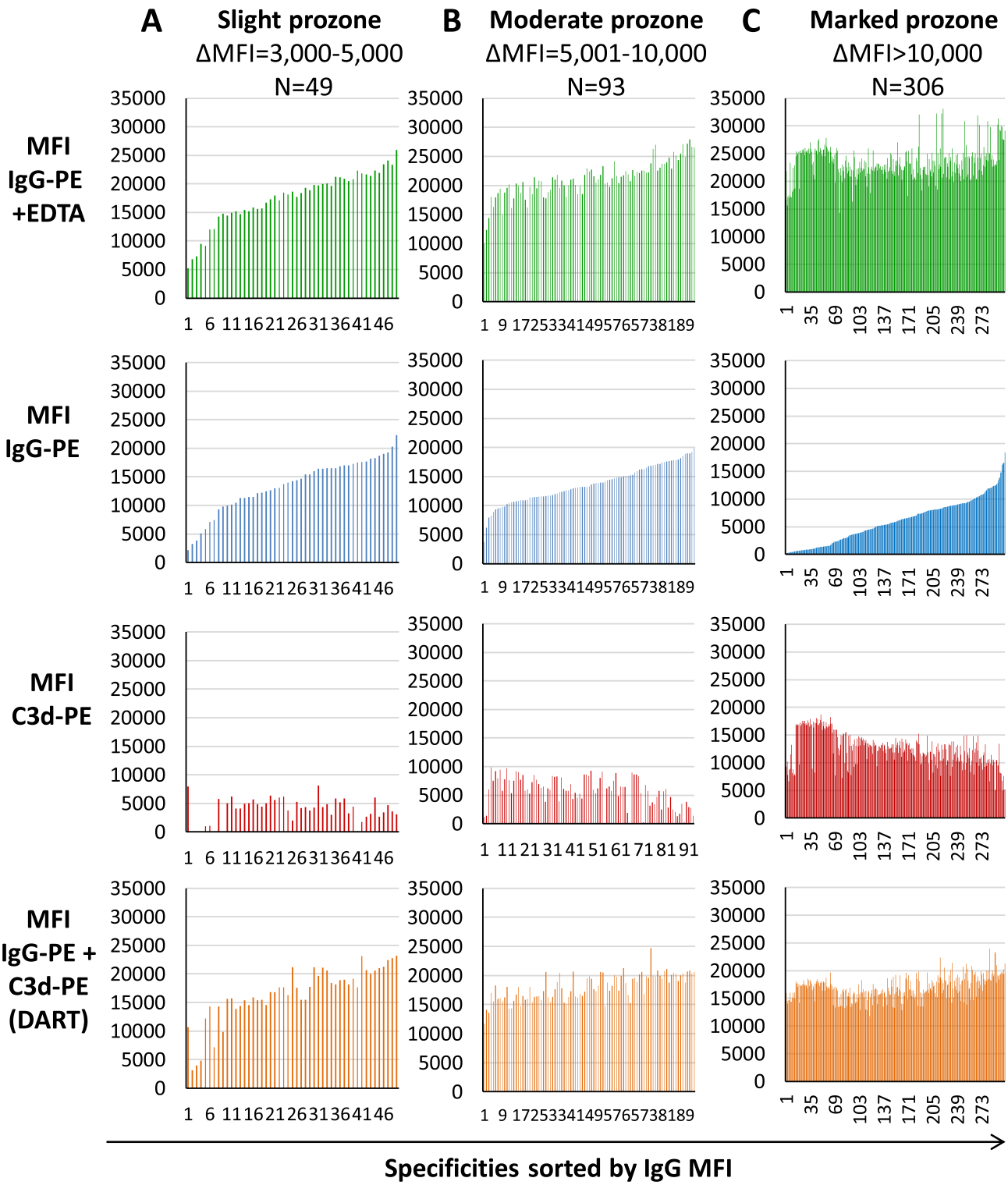

Fig. 4. Reactivity patterns of prozone positive specificities. SAB reactivity of 20 prozone positive patient sera tested with the IgG-PE ± EDTA, C3d-PE, and the DART protocols. Data depicts specificities exhibiting either slight (panel A; ΔMFI: 3000–5000), moderate (panel B; ΔMFI: 5001–10,000) or marked (panel C; ΔMFI > 10,000) prozone. ΔMFI = IgG-PE + EDTA MFI – IgG-PE MFI for each specificity. All specificities are sorted by IgG-PE MFI.

3. Results

3.1. Hypothesis and experimental design

Recent studies have demonstrated that the prozone-like effect, which compromises the detection of HLA antibodies in the SAB assay, is due to complement activation and deposition of complement split products (predominantly from C3 and C4) on the bead-bound HLA antigen/antibody complexes and subsequent interference with the binding of anti-IgG-PE reporter antibody [21–24]. Therefore, we hypothesized that:

1) HLA antibodies that exhibit the prozone effect fix large amounts of C3 split products on the bead-bound HLA antigen/antibody

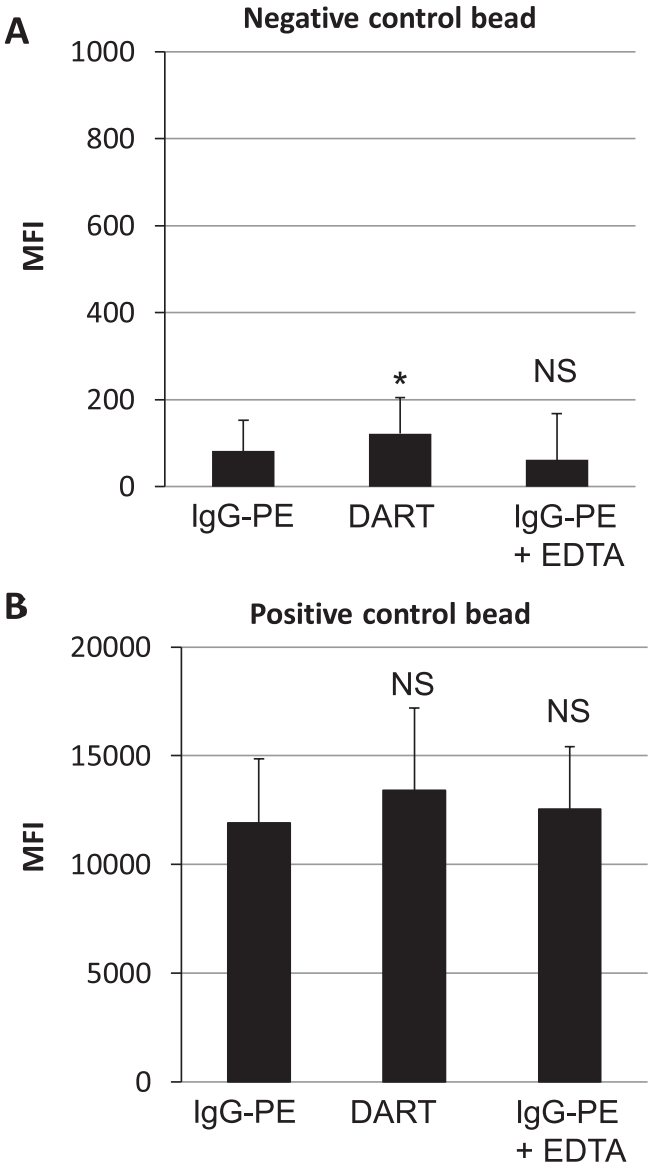

Fig. 5. Negative and positive control bead reactivity. Negative control and positive control bead reactivity for the IgG-PE ± EDTA and the DART protocols of 20 prozone positive patient sera (10 Class I and 10 Class II positive). Data depicts the mean ± SD of negative control bead MFI (panel A) and positive control bead MFI (panel B). "* " indicates $p < 0.05$ (repeated measures ANOVA); "NS" = non significant.

complexes and therefore, should be detectable using the C3d-PE reporter antibody.

2) Use of a cocktail containing both IgG-PE and C3d-PE reporter antibodies in the SAB assay will allow the detection of both prozone negative (IgG ± C3d) and prozone positive (C3d ± IgG) HLA antibody specificities.

To test these hypotheses, we initially selected two prozone positive sera (one class I and one class II HLA) and tested them using variations of the ROB protocol with three different reporter antibodies: 1) IgG-PE alone, 2) C3d-PE alone, and 3) a combination of IgG-PE and C3d-PE (DART protocol). To highlight prozone positive specificities, sera were tested using the IgG-PE reporter protocol with and without EDTA treatment. Using the same experimental approach we subsequently tested 18 additional prozone positive sera and performed a detailed comparison of reactivity patterns of prozone positive and prozone negative HLA antibody specificities tested with the different protocols.

3.2. Prozone positive specificities are detected using the C3d-PE or the combination of IgG-PE and C3d-PE reporter antibodies (DART protocol)

Fig. 1A shows the class I HLA antibody reactivity pattern of a prozone positive serum tested using the ROB protocol with the IgG-PE reporter antibody. Serum treatment with EDTA (Fig. 1B) highlights numerous prozone positive specificities (indicated by black rectangles), which are characterized by an increase in MFI (> 5000) compared to the untreated serum (Fig. 1B vs 1A). HLA antibody specificities outside of black rectangles are either IgG negative (MFI < 1000) or exhibit similar MFI values regardless of EDTA treatment and are therefore considered prozone negative (Fig. 1A and 1B). Interestingly, all prozone positive specificities were detected with the C3d-PE reporter antibody, showing C3d MFI values ranging between 5000 and 15,000 (Fig. 1C). In contrast, most prozone negative reactions showed virtually no staining with C3d-PE with the exception of three specificities (indicated by blue arrows, C3d MFI 1500–5000; Fig. 1C), all of which exhibited high IgG MFI values (> 10,000; Fig. 1A, blue arrows). Finally, no reactivity with C3d-PE was observed for IgG negative (IgG MFI < 1000) specificities (Fig. 1C vs A).

The results of the DART protocol testing are shown in Fig. 1D and demonstrate that all prozone positive specificities (black rectangles) are identified by combined IgG-PE and C3d-PE reporter antibody staining with a substantial overall increase in MFI values (DART MFI: 15,000–18,000) compared to IgG-PE reporter staining alone (Fig. 1A). Nevertheless, the increase in MFI values for prozone positive specificities seen with the DART protocol were slightly lower than those seen with the EDTA treatment (EDTA MFI: 17,000–24,000; Fig. 1B). Importantly, DART protocol MFI values for the prozone negative specificities were similar to those obtained with the IgG-PE reporter alone and no reactivity was observed for IgG negative specificities when DART protocol was used (Fig. 1A vs D).

The reactivity patterns of a second prozone positive serum tested against class II HLA SABs is shown in Fig. 2. The overall trends were similar to those described for class I HLA testing and show that all prozone positive specificities (> 5000 MFI increase with EDTA; black rectangles; Fig. 2A and B) are detected by the C3d-PE reporter antibody (Fig. 2C) and exhibit a substantial increase in MFI values, compared to IgG-PE alone (Fig. 2A), when tested using the DART protocol (Fig. 2D). No C3d-PE reporter antibody staining was seen for IgG negative or prozone negative specificities in this serum (Fig. 2C and D vs A). Finally, DART protocol MFI values for prozone negative specificities were again similar to those seen with IgG-PE alone (Fig. 2D vs A).

3.3. Detailed analysis of prozone negative and prozone positive HLA antibody reactivity patterns, a comparison between the IgG-PE ± EDTA, C3d-PE and DART protocols

Seeing the effectiveness of the C3d-PE reporter antibody and the DART protocol staining in detecting prozone positive specificities we decided to extend our studies by testing a total of 20 prozone positive sera (10 class I and 10 class II HLA). As before all sera were tested using the ROB protocol with either the IgG-PE reporter antibody (± EDTA), C3d-PE reporter antibody, or both antibodies (DART protocol). All HLA specificities were sorted based on IgG-PE MFI (Figs. 3 and 4) and classified as follows: (1) IgG-negative (IgG-PE + EDTA MFI < 1000, n = 735; Fig. 3A), (2) prozone negative (ΔMFI with EDTA treatment < 3000, n = 737; Fig. 3B), (3) slight prozone (Δ MFI with EDTA treatment = 3000–5000, n = 49; Fig. 4A), (4) moderate prozone (Δ MFI with EDTA treatment = 5001–10,000, n = 93; Fig. 4B), and (5) marked prozone (Δ MFI with EDTA treatment > 10,001, n = 306; Fig. 4C).

Fig. 3A shows that all IgG negative specificities remained negative upon testing with either the C3d-PE reporter antibody alone or with the DART protocol. In contrast, 107 out of 737 (14.5%) of prozone negative specificities demonstrated positive (> 1000 MFI) staining with the C3d-

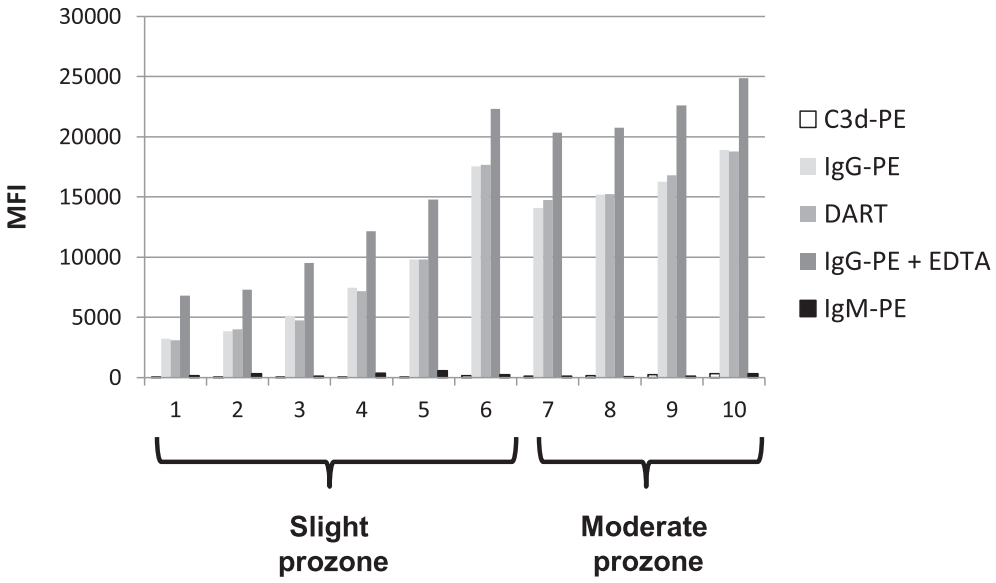

Fig. 6. Reactivity pattern of ten prozone positive specificities that do not fix complement (C3d negative). Data shows trimmed MFI values for each specificity obtained using the following protocols: C3d-PE, IgG-PE, DART, IgG-PE + EDTA and IgM-PE. Ten specificities that do not fix complement (C3d negative) and exhibit increased MFI upon treatment with EDTA are shown. All specificities are sorted by IgG MFI.

PE antibody, although the C3d-PE MFI values were relatively low (mean MFI = 2300.0 ± 1142.4 range = 1001–6500; Fig. 3B). Of these 107 C3d positive specificities, 95 (89%) were considered strong antibodies and had an IgG-PE MFI > 10,000. In fact, the probability of C3d positivity was directly proportional to the IgG-PE MFI and was 1.6% (IgG-PE MFI = 1000–5000), 5.3% (IgG-PE MFI = 5001–10,000), 25% (IgG-PE MFI = 10,001–15,000), 36% (IgG-PE MFI = 15,001–20,000), and 52% (IgG-PE MFI > 20,000). Importantly, DART protocol testing showed similar MFI to those seen with the IgG-PE reporter alone for prozone negative specificities ($R^2 = 0.97$; Fig. 3B). The correlation between IgG-PE and IgG-PE + EDTA protocols was slightly lower ($R^2 = 0.93$; Fig. 3B)

Fig. 4 shows that most prozone positive specificities (438 out of 448, 98%) fix complement and are detected with the C3d-PE reporter antibody (C3d-PE MFI > 1000). The probability of C3d positivity was 88%, 96%, and 100% for HLA specificities within the slight, moderate and marked prozone groups, respectively. Interestingly, C3d-PE MFI was directly proportional to the degree of prozone (mean C3d-PE MFI = 4419.5 ± 1606.3 for slight, 5991.0 ± 2302.7 for moderate, and 12,417.4 ± 2969.9 for marked prozone specificities). Importantly, the DART protocol detected all prozone positive reactions, MFI value ranges for the slight, moderate and marked prozone specificities were 3083.1–23,228.9, 11,671.2–24,749, and 11,757.2–23,984.6, respectively (Fig. 4). The average DART MFI increase from IgG-PE MFI for prozone positive specificities was lower compared to EDTA treatment (mean Δ MFI of 3022 ± 2072 vs 4099 ± 606.8 for slight prozone; 4343.6 ± 2112.6 vs 7418.9 ± 1437.8 for moderate prozone; and 10,911.1 ± 3486.6 vs 17,357.7 ± 4273.5 for marked prozone). However, all moderate and marked prozone specificities with IgG-PE MFI < 5000 (ie. specificities that may be miss-assigned as negative depending on the SAB assay positive cutoff used) exhibited greatly increased MFI values with the DART protocol ranging from 11,671.2 to 19,695.1 (mean Δ MFI = 13,911.96 ± 2551.5), and would not be in danger of being miss-assigned as negative.

Finally, we analysed MFI values for the negative (Fig. 5A) and positive (Fig. 5B) control beads to determine if inclusion of the C3d-PE reporter antibody in the DART protocol had an impact on the background or IgG-PE reactivity in the SAB assay. While there was a statistically significant difference between the average MFI value of negative control bead for the IgG-PE vs DART protocols ($p < 0.05$; repeated measures ANOVA), the differences were minor (mean MFI = 81.8 ± 83.6 vs 121.5 ± 107.1 for IgG-PE vs DART) and would not significantly affect SAB assay results or interpretation. Positive control beads in the SAB assay kits are pre-coated with human IgG and are used as a control for IgG-PE antibody binding. No statistically significant differences in positive control bead MFI were detected between the three protocols (Fig. 5B).

3.4. Possible off target effects of EDTA treatment on HLA antibody binding/detection.

Further analysis of HLA antibody reactivity patterns described in the previous section raised the possibility that EDTA treatment may have off target effects on HLA antibody binding and/or detection. Two examples are described below:

1) We identified a total of ten C3d negative specificities that were assigned to either the slight (n = 6) or moderate (n = 4) prozone groups based on the MFI differential between the untreated and EDTA treated samples (Fig. 6). DART MFI values for these specificities were comparable to IgG-PE MFI (Fig. 6) confirming that the prozone like effect observed for these specificities was not due to complement mediated interference. Furthermore, none of these specificities stained with the IgM-PE reporter antibody (IgM-PE MFI < 400; Fig. 6) which ruled out the possibility that IgM alloantibody-mediated interference was responsible for the effect. Taken together these findings suggest the possibility of an off target effect of EDTA treatment leading to enhanced HLA alloantibody binding or detection.
2) Upon further analysis of prozone-negative specificities (Fig. 3B), we identified a subpopulation of reactivities (n = 110) showing reduced MFI values following treatment with EDTA (> 2000 MFI reduction compared to untreated IgG-PE group; Fig. 7A). This suggests that EDTA treatment may have a negative effect on the binding of some HLA antibodies in the SAB assay. These specificities represent approximately 15% of the IgG positive, prozone-negative reactions and were present in 9 out of the 20 sera tested (45%). For these specificities, IgG-PE MFI correlated better with DART MFI than with IgG-PE + EDTA MFI ($R^2 = 0.95$ and $R^2 = 0.89$, respectively; Fig. 7B). In fact, the correlation was stronger for IgG-PE vs DART MFI (Fig. 8A) compared to IgG-PE vs IgG-PE + EDTA MFI (Fig. 8B) for prozone negative specificities ($R^2 = 0.97$ and $R^2 = 0.93$, respectively; Fig. 8).

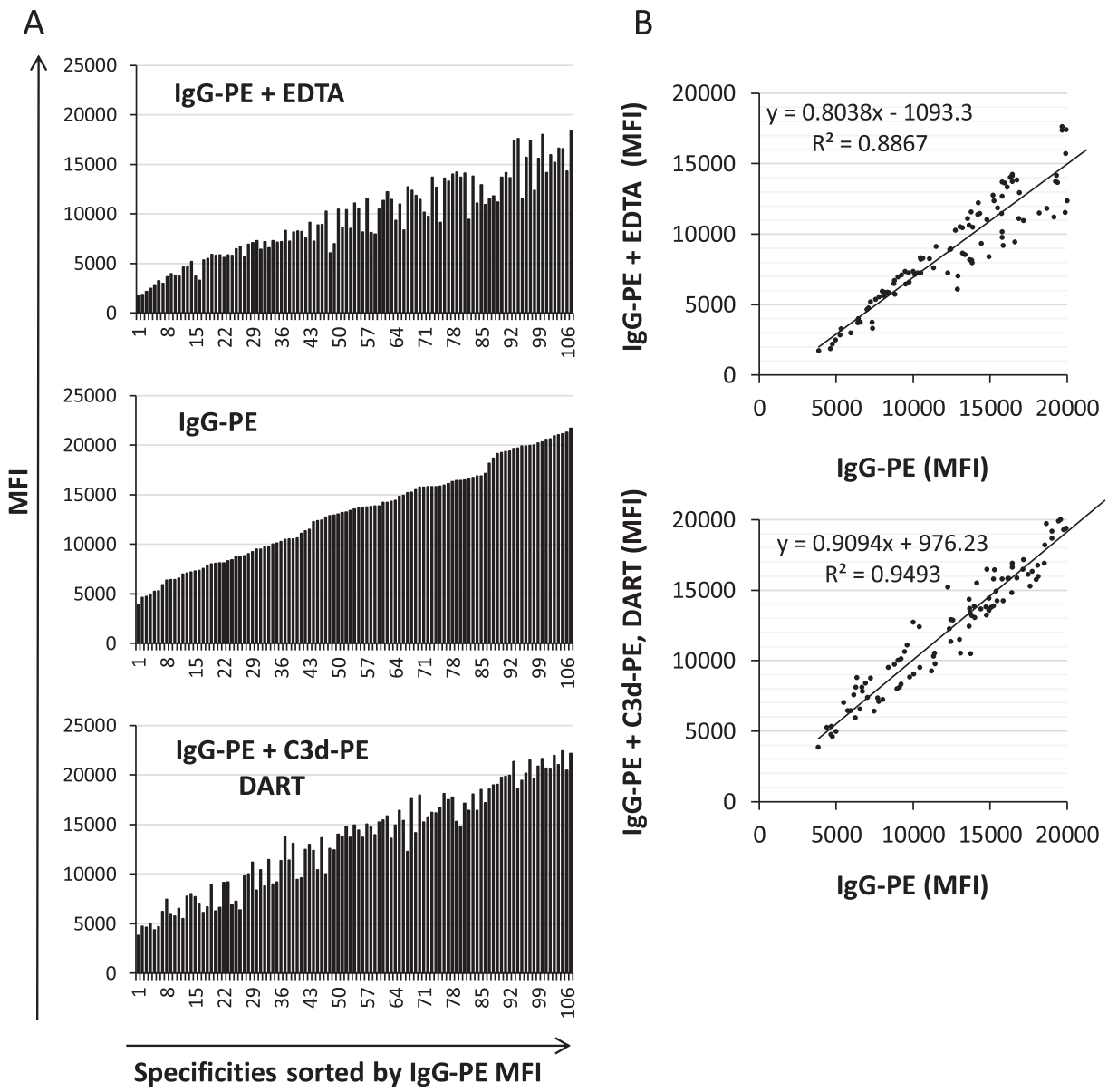

Fig. 7. EDTA treatment can negatively impact MFI of some IgG positive specificities. Reactivity pattern (IgG-PE ± EDTA and the DART protocols) of prozone negative specificities with ΔMFI > 2000 (IgG-PE MFI – IgG-PE + EDTA MFI; n = 110). Panel A shows trimmed MFI values for the prozone negative specificities tested by IgG-PE + EDTA (top panel), IgG-PE (middle panel) and DART (bottom panel) protocols. All specificities are sorted by IgG-PE MFI. Panel B shows linear regression and R^2 values for IgG-PE vs IgG-PE + EDTA MFI (top panel) and IgG-PE vs DART MFI (bottom panel).

4. Discussion

The goal of this study was to develop a SAB assay protocol that can detect HLA antibodies independent of the prozone effect, which is predominantly due to complement mediated interference with anti-IgG-PE reporter antibody binding to HLA alloantibodies on SABs [21–24]. To this end we followed a simple "if you can't beat them join them" approach, and rather than trying to inhibit complement activation by treating sera using modalities such as heat, DTT, or EDTA, we used it to our advantage and added the anti-C3d-PE reporter antibody in order to detect C3d deposition on complement fixing HLA alloantibodies in the SAB assay. The resulting DART SAB protocol detects both prozone negative and prozone positive HLA alloantibodies by using a cocktail of IgG-PE and C3d-PE reporter antibodies. The DART method is based on our recently developed rapid optimized SAB (ROB) protocol [30], featuring shortened incubation and centrifugation times, and can therefore be performed in approximately 30 min. This represents a 60–70% time reduction compared to the standard LABScreen SAB assay. Parallel testing of 20 prozone positive sera demonstrated that the DART protocol generates virtually identical results to the standard IgG-PE reporter ROB protocol for IgG negative and prozone negative reactions. Specifically, there were no false positive reactions noted and the MFI correlation between the DART and IgG-PE protocols for prozone negative specificities was excellent (R^2 = 0.97). In fact it was slightly improved when compared to the treatment with EDTA (R^2 = 0.93). Importantly, the DART protocol detected and significantly increased the MFI values for all marked prozone specificities and for the majority of reactivities classified as either moderate (96%) and slight (88%) prozone.

As mentioned above, we observed a small number of reactions (n = 10) classified into the moderate and slight prozone groups (based on increased MFI with EDTA) that did not exhibit increased MFI upon testing with the DART protocol. None of these specificities showed any C3d or IgM staining, ruling out the presence of either complement or

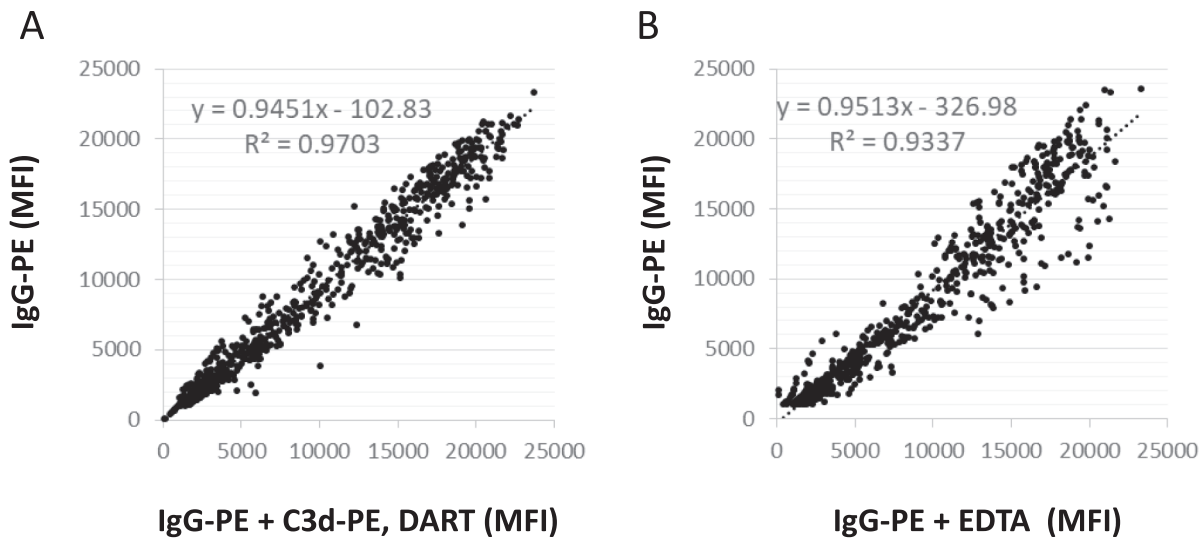

Fig. 8. DART MFI correlates better with IgG-PE MFI than IgG-PE + EDTA MFI for prozone negative specificities. Reactivity pattern (IgG-PE ± EDTA and the DART protocols) of prozone negative specificities. Panel A shows linear regression and R^2 values for IgG-PE vs DART MFI correlation. Panel B shows linear regression and R^2 values for IgG-PE vs IgG-PE + EDTA MFI correlation.

IgM mediated interference. This suggests that the increase in MFI values seen with EDTA treatment for some HLA antibody specificities may in fact be artefactual. We also identified 110 reactions (15% of prozone negative specificities) with decreased MFI values (by more than 2000) following treatment with EDTA. The reduction in MFI noted for these specificities did not lead to changes in the antibody assignment (positive vs negative) based on a 1000 MFI cutoff used in the Halifax HLA laboratory. However, if the positive threshold was changed to either 2000 or 3000 MFI (used by many HLA laboratories in the USA) several of the "positive" specificities could be reassigned to "negative". Importantly, the MFI correlation between the DART and IgG-PE protocols for these specificities was improved when compared to EDTA (R^2 = 0.95 vs R^2 = 0.89), with no specificities falling below the 2000 or 3000 MFI threshold when tested with the DART protocol. Therefore, the DART protocol may be a good alternative to serum treatment procedure as it is resistant to prozone but at the same time it is not susceptible to artefacts related to serum treatment. Differences in SAB reactivity between treated and untreated sera have also been seen in other studies. Guidicelli et al. (2018) found 8.1% of class I and 15.9% of class II reactivities, that were not determined to be affected by prozone, differed in their final assignment depending on the absence or presence of EDTA [31]. Meanwhile a separate study saw a slight increase in MFI in a subset of reactivities from prozone negative sera treated with DTT compared to untreated sera [29]. The mechanism underlying these serum treatment artefacts remain unclear and require further study.

The MFI increase seen with the DART protocol for prozone positive specificities was less pronounced (by approximately 25–30%) compared to that observed with EDTA treatment. However, all moderate and marked prozone specificities with IgG-PE MFI < 5000, exhibited greatly increased MFI values with the DART protocol (mean Δ MFI = 13,911.96 ± 2551; range = 11,671.2–19,695.1) and would clearly be identified and assigned as unacceptable HLA antigens. Based on the review of recent studies [29,35] showing EDTA to be significantly more effective than DTT (as low as 47% effectiveness in some cases) [29] at reversing prozone, we believe that the DART protocol is superior to DTT with respect to the increase in MFI for prozone positive specificities. Our future studies will determine if using higher concentration of C3d-PE antibody in the DART protocol, or a longer incubation time, may enhance the Δ MFI for prozone positive specificities to levels obtained with the EDTA treatment.

Our observation that the majority of prozone positive specificities are C3d positive and fix complement is consistent with other studies that identified complement mediated interference as the main mechanism for the prozone effect in the SAB assay [21,22,24]. Interestingly, the C3d-PE MFI values were proportional to the degree of prozone. Thus, the extent of interference appears to be directly related to the amount of complement activated and deposited on the HLA alloantibody. Only 10 out of 448 specificities that were assigned as prozone positive based on MFI increase with EDTA treatment did not show any C3d deposition. As explained earlier, there was no significant anti-HLA IgM component to explain the interference in any of these cases leading us to believe that the EDTA treatment related MFI increase represents an off target effect of EDTA.

It is interesting to note that some C3d staining was observed for a number of prozone negative specificities. The C3d MFI values for these specificities were significantly lower compared to prozone positive specificities suggesting that the amount of C3d deposited was below the level required to effectively prevent HLA antibody detection by the IgG-PE reporter and thus induce a significant prozone effect. The likelihood and the degree (MFI) of C3d positivity for prozone negative specificities correlated directly with IgG-PE MFI. This is in agreement with the results reported by studies using the C1q assay or the traditional C3d assay [32–34] to detect complement binding/fixing antibodies. Although we have yet to directly compare our modified ROB protocol using the C3d-PE reporter antibody to traditional complement detecting assays currently on the market, we speculate that our protocol may provide similar results using a more simple and rapid methodology.

In summary, the SAB assay plays a key role in alloantibody detection and identification for the assessment of immunologic risk in organ transplantation. The prozone effect remains one of the most concerning limitations of this assay, and although serum treatment with EDTA can effectively inhibit complement activation and abrogate prozone, there are still concerns that it may also possess off target effects. Thus, implementation of the DART protocol may be an easy way to overcome the prozone effect without compromising HLA antibody detection.

Conflicts of interest

The authors declare they have no conflicts of interest.

Acknowledgments

The authors gratefully acknowledge the technical assistance of the Halifax HLA Laboratory technologists, especially Mr. Geoff Adams

(MLT) and Mrs. Kelly Heinstein (BSc, MLT, HLA lab supervisor). This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

[1] R. Pei, J.H. Lee, N.J. Shih, M. Chen, P.I. Terasaki, Single human leukocyte antigen flow cytometry beads for accurate identification of human leukocyte antigen antibody specificities, Transplantation 75 (2003) 43–49.

[2] One Lambda Inc., LABScreen Single Antigen HLA class I and class II, product insert, (n.d.). http://www.onelambda.com/.

[3] J.M. Cecka, Current methodologies for detecting sensitization to HLA antigens, Curr. Opin. Organ Transplant. 16 (2011) 398–403.

[4] H.M. Gebel, R.A. Bray, The evolution and clinical impact of human leukocyte antigen technology, Curr. Opin. Nephrol. Hypertens. 19 (2010) 598–602.

[5] R.A. Bray, J.D.L. Nolen, C. Larsen, T. Pearson, K.A. Newell, K. Kokko, A. Guasch, P. Tso, J.B. Mendel, H.M. Gebel, Transplanting the highly sensitized patient: the Emory algorithm, Am. J. Transplant. 6 (2006) 2307–2315.

[6] C.J. Taylor, V. Kosmoliaptsis, L.D. Sharples, D. Prezzi, C.H. Morgan, T. Key, A.N. Chaudhry, I. Amin, M.R. Clatworthy, A.J. Butler, C.J.E. Watson, J.A. Bradley, Ten-year experience of selective omission of the pretransplant crossmatch test in deceased donor kidney transplantation, Transplantation 89 (2010) 185–193.

[7] A.W. Bingaman, C.L. Murphey, J. Palma-Vargas, F. Wright, A virtual crossmatch protocol significantly increases access of highly sensitized patients to deceased donor kidney transplantation, Transplantation 86 (2008) 1864–1868.

[8] J.M. Cecka, A.Y. Kucheryavaya, N.L. Reinsmoen, M.S. Leffell, Calculated PRA: initial results show benefits for sensitized patients and a reduction in positive crossmatches, Am. J. Transplant. Transpl. Surg. 11 (2011) 719–724.

[9] L.A. Baxter-Lowe, M. Cecka, M. Kamoun, J. Sinacore, M.L. Melcher, Center-defined unacceptable HLA antigens facilitate transplants for sensitized patients in a multicenter kidney exchange program, Am. J. Transplant. 14 (2014) 1592–1598.

[10] P. Amico, G. Hönger, J. Steiger, S. Schaub, Utility of the virtual crossmatch in solid organ transplantation, Curr. Opin. Organ Transplant. 14 (2009) 656–661.

[11] C. Lefaucheur, A. Loupy, G.S. Hill, J. Andrade, D. Nochy, C. Antoine, C. Gautreau, D. Charron, D. Glotz, C. Suberbielle-Boissel, Preexisting donor-specific HLA antibodies predict outcome in kidney transplantation, J. Am. Soc. Nephrol. 21 (2010) 1398–1406.

[12] C. Wiebe, I.W. Gibson, T.D. Blydt-Hansen, M. Karpinski, J. Ho, L.J. Storsley, A. Goldberg, P.E. Birk, D.N. Rush, P.W. Nickerson, Evolution and clinical pathologic correlations of de novo donor-specific HLA antibody post kidney transplant, Am. J. Transplant. 12 (2012) 1157–1167.

[13] C. Wiebe, I.W. Gibson, T.D. Blydt-Hansen, D. Pochinco, P.E. Birk, J. Ho, M. Karpinski, A. Goldberg, L. Storsley, D.N. Rush, P.W. Nickerson, Rates and determinants of progression to graft failure in kidney allograft recipients with de novo donor-specific antibody, Am. J. Transplant. 15 (2015) 2921–2930.

[14] H.M. Gebel, R.S. Liwski, R.A. Bray, Technical aspects of HLA antibody testing, Curr. Opin. Transplant. 18 (2013) 455–462.

[15] T. Roberts, G. Tumer, H.M. Gebel, R.A. Bray, Solid-phase assays for the detection of alloantibody against human leukocyte antigens: panacea or pandora? Int. J. Immunogenet. 41 (2014) 362–369.

[16] H.M. Gebel, R.A. Bray, HLA antibody detection with solid phase assays: great expectations or expectations too great? Am. J. Transplant. 14 (2014) 1964–1975.

[17] R.S. Liwski, H.M. Gebel, Of cells and microparticles: assets and liabilities of HLA antibody detection, Transplantation 102 (2018) S1–S6.

[18] A.A. Zachary, D.P. Lucas, B. Detrick, M.S. Leffell, Naturally occurring interference in Luminex assays for HLA-specific antibodies: characteristics and resolution, Hum. Immunol. 70 (2009) 496–501.

[19] V. Kosmoliaptsis, C. O'Rourke, J.A. Bradley, C.J. Taylor, Improved Luminex-based human leukocyte antigen-specific antibody screening using dithiothreitol-treated sera, Hum. Immunol. 71 (2010) 45–49.

[20] J.F.M. Jacobs, R.G. Van Der Molen, X. Bossuyt, J. Damoiseaux, Antigen excess in modern immunoassays: to anticipate on the unexpected, Autoimmun. Rev. 14 (2015) 160–167.

[21] M. Schnaidt, C. Weinstock, M. Jurisic, B. Schmid-horch, A. Ender, D. Wernet, HLA antibody specification using single-antigen beads — a technical solution for the prozone effect, Transplantation 92 (2011) 510–515.

[22] C. Weinstock, M. Schnaidt, The complement-mediated prozone effect in the Luminex single-antigen bead assay and its impact on HLA antibody determination in patient sera, Int. J. Immunogenet. 40 (2013) 171–177.

[23] J. Visentin, M. Vigata, S. Daburon, C. Contin-Bordes, V. Fremeaux-Bacchi, C. Dromer, M.-A. Billes, M. Neau-Cransac, G. Guidicelli, J.-L. Taupin, Deciphering complement interference in anti-human leukocyte antigen antibody detection with flow beads assays, Transplantation 98 (2014) 625–631.

[24] E. Schwaiger, M. Wahrmann, G. Bond, F. Eskandary, G.A. Bohmig, Complement component C3 activation: the leading cause of the prozone phenomenon affecting HLA antibody detection on single-antigen beads, Transplantation 97 (2014) 1279–1285.

[25] R. Liwski, R. Bray, H. Gebel, Keep it cool. A novel inhibitor complex exclusion (ICE) protocol for LABScreen that prevents the "prozone" effect, Hum. Immunol. 75 (2014) 7.

[26] R.S. Liwski, R.A. Bray, H.M. Gebel, Keep it cool: a novel inhibitor complex exclusion (ICE) protocol for LABScreen that prevents the "prozone" effect, ASHI Q. (2016) 36–43.

[27] A.R. Tambur, N.D. Herrera, K.M.K. Haarberg, M.F. Cusick, R.A. Gordon, J.R. Leventhal, J.J. Friedewald, D. Glotz, Assessing antibody strength: comparison of MFI, C1q, and titer information, Am. J. Transplant. 15 (2015) 2421–2430.

[28] A. Zeevi, J. Lunz, B. Feingold, M. Shullo, C. Bermudez, J. Teuteberg, S. Webber, Persistent strong anti-HLA antibody at high titer is complement binding and associated with increased risk of antibody-mediated rejection in heart transplant recipients, J. Hear. Lung Transplant. 32 (2013) 98–105.

[29] J. Wang, J. Meade, N. Brown, J. Weidner, S. Marino, EDTA is superior to DTT treatment for overcoming the prozone effect in HLA antibody testing, HLA. 89 (2017) 82–89.

[30] R.S. Liwski, A.L. Greenshields, C. Murphey, R.A. Bray, H.M. Gebel, It's about time: the development and validation of a rapid optimized single antigen bead (ROB) assay protocol for LABScreen, Hum. Immunol. 78 (2017).

[31] G. Guidicelli, J. Visentin, N. Franchini, C. Borg, P. Merville, L. Couzi, J.L. Taupin, Prevalence, distribution and amplitude of the complement interference phenomenon in single antigen flow beads assays, HLA. 91 (2018) 507–513.

[32] S. Schaub, G. Hönger, M.T. Koller, R. Liwski, P. Amico, Determinants of C1q binding in the single antigen bead assay, Transplantation 98 (2014) 387–393.

[33] G. Claisse, L. Absi, F. Cognasse, E. Alamartine, C. Mariat, N. Maillard, Relationship between mean fluorescence intensity and C1q/C3d-fixing capacities of anti-HLA antibodies, Hum. Immunol. 78 (2017) 336–341.

[34] G. Claisse, C. Mariat, N. Maillard, From accurate assessment of anti-HLA antibody MFI to complement-binding assays, Clin. Transpl. 32 (2016) 153–160 (accessed October 30, 2018), http://www.ncbi.nlm.nih.gov/pubmed/28564533.

[35] X. Zhang, N.L. Reinsmoen, Comprehensive assessment for serum treatment for single antigen test for detection of HLA antibodies, Hum. Immunol. 78 (2017) 699–703.