Human Immunology 76 (2015) 849–857

Contents lists available at ScienceDirect

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

Improved flow cytometry based cytotoxicity and binding assay for clinical antibody HLA crossmatching

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ARTICLE INFO

Article history:
Received 16 May 2014
Revised 2 September 2014
Accepted 28 September 2015
Available online 30 September 2015

Keywords:
HLA antibodies
Transplantation
Complement-dependent lymphocytotoxicity
Flow cytometry crossmatch
C1q assay

ABSTRACT

The presence of preformed donor-specific HLA antibodies leads to early antibody mediated kidney allograft rejection. Therefore, detection and avoidance of donor reactive HLA antibodies prior to transplantation is of outmost importance in order to minimize the risk of rejection. Detection of pre-formed HLA antibodies is currently performed using complement-dependent cytotoxicity (CDC) assay alone or together with a flow cytometry based crossmatch (FCXM). This study was initiated to further evaluate our recently developed flow cytometry based procedure for determination of both cytotoxicity of and IgG binding to donor-derived lymphocytes by HLA antibodies. Highly enriched immuno-magnetic bead purified T and B lymphocytes were used as target cells for patient sera using 96-well plates. Importantly, the assay shows high sensitivity and specificity as determined by HLA typed donor cells and serum with defined HLA antibody IgG and C1q. Based on this and additional data generated in this paper, such as evaluation of appropriate serum and complements incubation times and assay reproducibility and stability, will enable us to more rapidly implement this assay in our clinical laboratory routines. In addition, we demonstrate that FCtox crossmatching of deceased donor cells has superior specificity compared to conventional CDC assay especially regarding high frequencies of false-positive reactions.

1. Introduction

Patients with terminal chronic kidney failure are often in need of life-long dialysis in addition to medical treatment. Reduced quality of life and increased risk of mortality when in dialysis will eventually require kidney transplantation. However, pre-formed donor specific HLA antibodies (DSA) induced by previous sensitizing events (e.g. pregnancies, blood transfusions or transplantations) may cause early antibody mediated allograft rejection [1–3]. In order to minimize the risk of rejection, transplantation laboratories of today have a set of tools to determine DSA and compatibility of the patient and donor. There are two major cell-based *in vitro* laboratory techniques available for detection of DSA; (i) complement-dependent cytotoxicity (CDC) assays [4] and (ii) flow cytometric crossmatch (FCXM) assays [5,6]. It is well established that CDC positive crossmatches (due to preformed DSA) are associated with early antibody mediated rejection (AMR) and allograft loss [1]. However, the CDC assay detects only complement fixing antibodies and there are weaknesses associated with this assay such as insensitivity and non-specific responses. DSA can be detected with higher sensitivity by using the flow cytometry based crossmatch (FCXM) assay [7]. Importantly, low titer antibodies unrecognized by CDC can be detected by FCXM [8,9]. These FCXM+/CDC− antibodies are clinically relevant and if recognized prior to transplantation rejection can be avoided by selecting an alternative donor or by desensitization of the patient (e.g. immunoadsorption and/or plasmapheresis) [10]. One drawback with the FCXM assay is that it does not permit discrimination of complement versus non-complement fixing HLA antibodies. Therefore some efforts have been made on combining the determination of antibody binding and cytotoxicity using flow cytometry based assays [11–13]. Recently we published a paper describing an alternative novel assay using pre-sorted T and B cells as donor cells [14]. The aim of the present study was to further optimize this protocol and adapt it to routine laboratory conditions as well as critically evaluate its performance and robustness. We present FCtox data on deceased donor crossmatching in comparison with conventional CDC. In addition, selection of appropriate

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http://dx.doi.org/10.1016/j.humimm.2015.09.047
0198-8859/

target cell numbers, choice of serum/complement incubation times and evaluation of sample storage are presented.

2. Materials and methods

2.1. Isolation of peripheral blood mononuclear cells, HLA typing and serum antibody characterization

Whole blood was obtained from healthy volunteer donors and from the regular routine testing scheme of donors within the transplantation center at Karolinska University Hosptital in Sweden. All HLA antibody positive and negative test sera were from kidney patients. Negative control sera were obtained from healthy male AB negative blood donors and included in all crossmatches. The HLA antibody positive control serum was prepared by pooling sera from highly immunized patients with HLA class I and/or class II antibodies. In the deceased donor crossmatching DSA negative and positive sera with or without CDC reactivity were selected for FCtox assay. The antibody specificities of patient sera was determined by Luminex based Labscreen (LS) IgG assay and C1q (One Lambda, Canoga Park, LA) as a part of the regular routine testing conducted at the laboratory. The PCR based HLA typing (A, B; DR, DQ) of cells were performed within regular testing of donors prior to transplantation.

2.2. Immuno-magnetic bead isolation of T and B cells

Human T cells (CD3+) and B cells (CD19+) were isolated from peripheral blood mononuclear cells (PBMC) by immune-magnetic bead (EasySep® Human cell Enrichment Kits #19051 and 19054; Stem Cell Technologies, Vancouver, BC, Canada) negative isolation technique (Fig 1A). The purity of sorted cells were evaluated by flow cytometry and the purity was generally >90% for both T and B cells.

2.3. Flow cytometric cytotoxic assay

Crossmatches were performed with pre-sorted T and B cells as described recently [14]. Briefly, 20 μL patient serum (neat or diluted), negative and positive control serum were added to a 96-well plate (Fig 1B). Ten microliter of pre-sorted T and B cells (5×10^3–5×10^4/well) were then added. The cell/serum mixture was incubated for 30 min (or extended time as indicated) at RT (+22 °C). Cells were then washed twice by adding 150 μL wash buffer (WB; PBS/0.1% BSA) and centrifuged for 5 min (420 RCF, +4 °C). Thereafter cells were incubated for 30 min (or extended time as indicated) at RT with 20 μL standard rabbit complement diluted in 1 mL distilled water (CL3111; Cedarlane Laboratories Ltd, Ontario, Canada). Cells were then washed twice as above. Thereafter, 20 μL of a 1:100 fold diluted fluorescein isothiocyanate (FITC)-conjugated F $(ab')_2$ goat anti-human IgG (109-096-098; Jackson Immuno Research Laboratories, West Grove, PA) was added and incubated for 20 min at 4 °C. After 20 min incubation, cells were washed twice as above and thereafter 100 μL of a 1:100 fold diluted 7-Aminoactinomycin D (7-AAD) solution (Beckman Coulter Company, Miami, FL) was added to each well. Cells were then transferred to the flow cytometry (FC) tubes and additional WB (250 μL) was added to each FC tube. Samples were acquired on a FC500 MCL (Beckman Coulter) or FACScan (BD Biosciences) flow cytometers.

2.4. FCtox data analysis

Cytotoxicity was determined by the percentages of 7-AAD+ cells (i.e. non-viable) and the antibody binding was determined by the median fluorescence intensity (MFI, linear channel values) of the FITC conjugated secondary antibody. Data analysis was performed with CXP software for FC500 and CELLQuest software for FACScan. Live and dead cells were gated on the forward scatter (FSC)/side

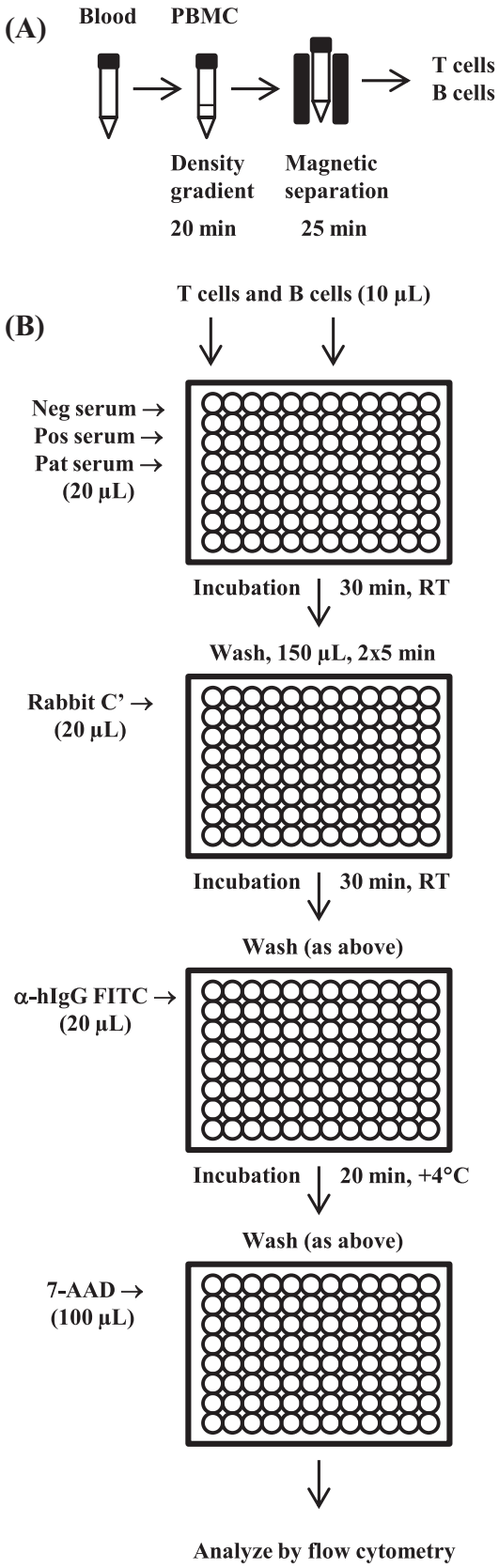

Fig. 1. Schematic diagram of FCtox protocol. (A) Immuno-magnetic bead isolation of T and B cells. (B) Flow cytometric cytotoxic assay.

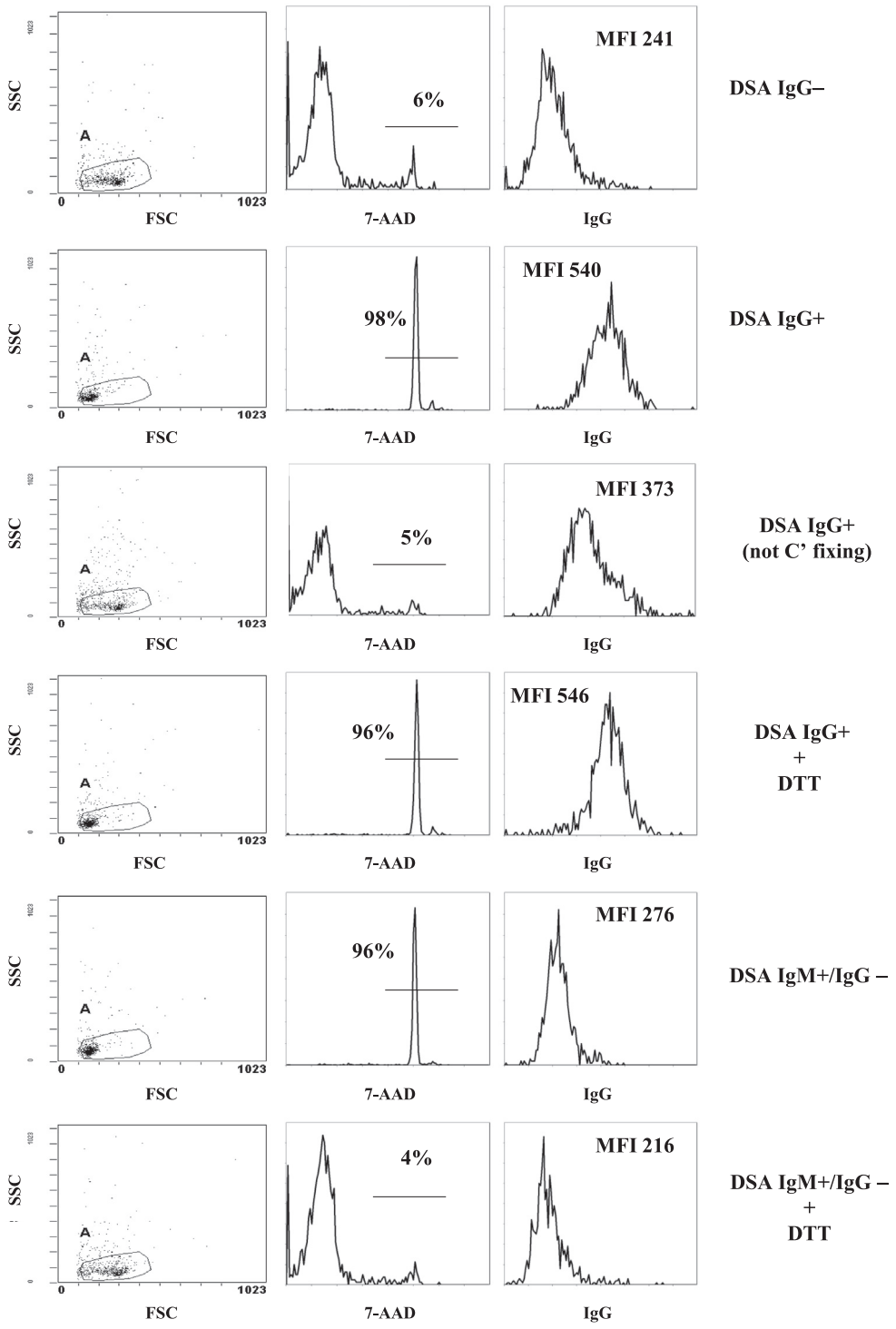

Fig. 2. Representative data plots from B cell FCtox crossmatch assay. 7-AAD staining (marker of cell viability) and antibody binding (IgG) on lymphocyte gated cells ("A" in FSC/SSC plot) were displayed in histograms. The mean fluorescence intensity (MFI) of IgG binding and percent 7-AAD+ cells is shown in plots. In indicated FCtox crossmatches sera were pre-treated with dithiothreitol (DTT) for 30 min at 37 °C to remove IgM reactivity.

scatter (SSC) plot (Fig 2). The percentages of dead cells (7-AAD+ cells) and levels of antibody binding on T and B cells were displayed in histograms. The percentage of cell lysis for each sample (Δ%) was calculated by subtracting the percentage of 7-AAD+ cells of negative control serum from the percentage of 7-AAD+ cells of sample. The test sample IgG channel shift (Δ MFI) was calculated by subtracting MFI value of negative control serum from sample MFI value.

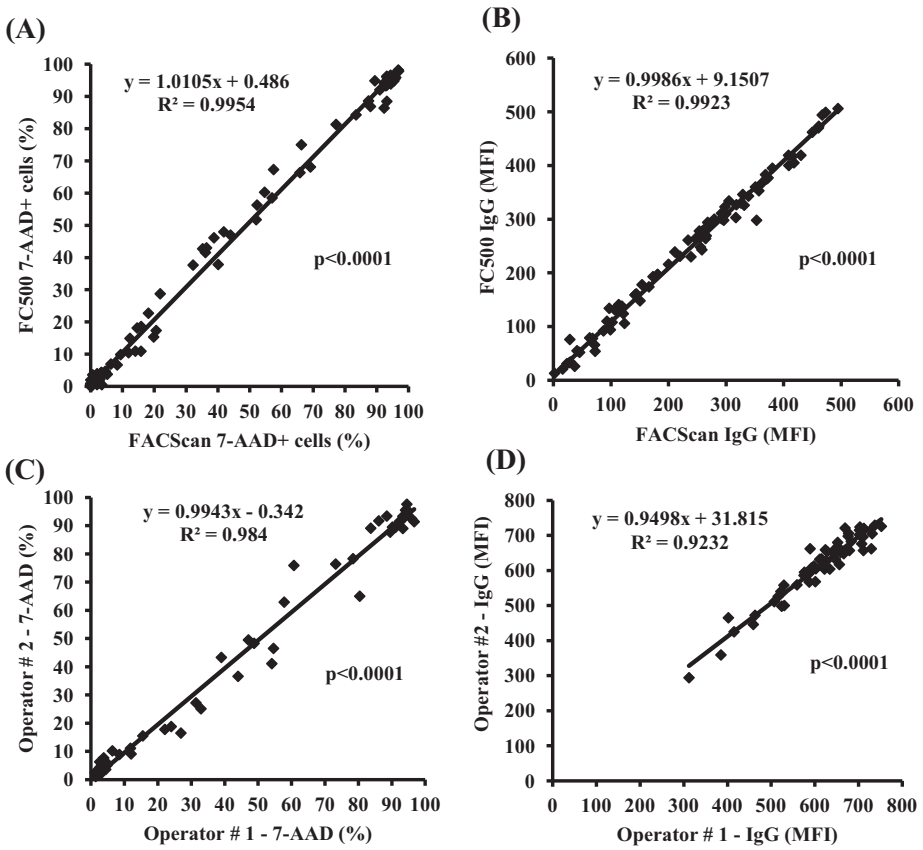

Fig. 3. Flow cytometry instrument and operator comparisons. (A) Relationship between FCtox 7-AAD and IgG data (serum/cell combinations, $n = 105$) generated by FC500 (Beckman Coulter) and FACScan (BD Biosciences) flow cytometers. (B) Relationship between FCtox 7-AAD and IgG data (serum/cell combinations, $n = 55$) generated independently by two operators. Note that the preparation of PBMC and cell sorting were performed by one of the operators. The p and R2 values are indicated in the graphs.

2.5. Complement-dependent cytotoxicity

T and B cells were isolated by positive selection from whole blood of donors by anti-CD8 (210.02D) and anti-CD19 (111.43D) conjugated magnetic beads (Life Technologies Corporation, Carlsbad, CA). Cells were incubated at room temperature for 30 min with 0.5 μL serum on Terasaki plates. Thereafter 2 μL of standard rabbit complement (CL3111; Cedarlane Laboratories Ltd, Ontario, Canada) and ethidium bromide/acridine orange mixture was added and incubated for 40 min at room temperature. In indicated experiments CDC crossmatching were performed with T and B cells isolated by negative selection as described in Section 2.2 (see above) and 30 min complement incubation time instead of 40 min. The following scoring system was used throughout the study; score 0 (0–10%) negative; score 2 (10–25%), 4 (25–50%), 6 (50–75%) and 8 (75–100%).

2.6. Statistical analyses

The two-tailed Fisher's exact test or Student's *t* test were used for statistical analysis. A P-value of <0.05 was considered significant. Statistical calculations were performed using Microsoft Excel Analyse-it® software (Microsoft Inc., Seattle, WA, USA).

3. Results

3.1. Evaluation of the FCtox assay robustness and flexibility

We have previously reported that the FCtox assay display low intra and inter-assay variation [14]. In order to further adapt the assay to clinical routine, we were interested in whether comparable results could be obtained using different flow cytometry platforms such as FACScan (BD Biosciences) and Cytomics FC500 (Beckman Coulter). We observed highly concordant data between instruments for both cytotoxicity (Fig 3A) and binding parameters (Fig 3B). Furthermore, low inter-operator variation was observed in crossmatches performed independently by two operators (Fig 3C and D). Importantly, samples were acquired and analyzed independently by two operators in order to further investigate inter-operator variations. In addition, three crossmatches done in parallel on cells from a given donor against a panel of sera confirmed that the FCtox assay display high degree of intra-assay consistency. Identical levels of lysis (Fig. 4A) and antibody binding (Fig 4B) were obtained and with similar degree of B cell purity (97, 96 and 98%) between cell isolations. In order to address whether delays in sample acquisition could affect the crossmatch results, samples were acquired immediately after completion of crossmatch assay and re-acquired after 2 h of storage in cold temperature (+4 °C). No significant changes in lysis (Fig 5A) or antibody binding (Fig 5B) was observed. Importantly, the level of background lysis or binding using negative serum (NS) was virtually unchanged after sample storage.

3.2. Optimization of target cell number/well and number of acquired cells/sample

In order to optimize assay requirements such as high sensitivity and short turn-around time we determined the minimal numbers of target cells/well, taking into account cell availability as well as length of acquisition time. Different numbers of pre-sorted T cells

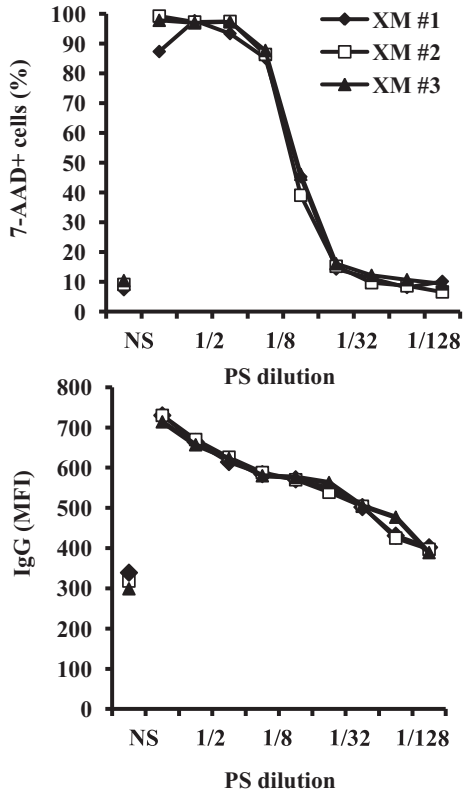

Fig. 4. Intra-assay comparisons. Three separate crossmatches (XM#1–3) were performed in parallel by one operator. The B cell purities were 97%, 96%, 98% for the separate crossmatches. Raw data 7-AAD and IgG values for negative serum (NS) and dilutions of HLA antibody positive sera (PS) were plotted. Similar data were obtained in one additional experiment.

were crossmatched against undiluted and several dilutions of HLA antibody positive serum (Fig 6). No significant changes in level of target lysis (Fig. 5A) or antibody binding (Fig 6B) were observed upon increased numbers of T cells/well. We found that B cell FCtox assay could be performed with as low as 5000 cells/well and no significant differences in cytotoxicity were observed upon increased cell numbers (Fig. 6C). Similar levels of intra-sample precisions were obtained at acquisition of 100, 500 or 1000 gated events per sample (Fig 6D). Acquisition of 100 events resulted in somewhat increased intra-sample variation at the low end scale for both the cytotoxicity and antibody binding parameter. However, the difference was not statistically significant. In most experiments throughout this study 1000 FSC/SSC gated events were acquired for each sample.

3.3. Evaluation of different serum and complement incubation time

In our initial evaluation study we observed that around 20% of the T cell crossmatches using DSA C1q+ sera did not result in positive T FCtox 7-AAD or CDC+ crossmatches [14]. Although the MFI levels of C1q DSA were relatively low (<3000) in the majority of these cases we here decided to evaluate whether extended serum and/or complement incubation times could increase the assay sensitivity. It was also of interest to investigate whether prolonged assay time would influence the overall performance of FCtox. Crossmatches using four different combinations of serum/complement incubation times (30 + 30, 30 + 45, 45 + 30 and 45 + 45 min) were performed with serially diluted HLA positive sera (Fig 7A and B). Highest cytotoxicity was observed when cells were incubated for 45 min with serum and 45 min with complement, followed by 45 + 30, 30 + 45 and lowest sensitivity was observed for 30 min serum and 30 min complement. There were no major differences in IgG binding between the combinations of serum/complement incubation times (Fig 7B). In separate experiments using B lymphocytes as targets increased level of lysis was observed upon extended serum or complement incubation times (data not shown). Noteworthy, extended complement time (45 or 60 min) significantly increased the level of background cytotoxicity. Using 30 min incubation resulted in background lysis of 5% ± 1 (n = 13) which increased to 11% ± 7 (n = 13) and 18% ± 11 (n = 13) at 45 and 60 min complement incubation, respectively (Fig 7C). In two crossmatches the background lysis increased from 7% (at 30 min) to 40% at 60 min incubation. No increase in background cytotoxicity was observed for T cells upon extended complement time (data not shown).

3.4. Comparison of assay sensitivity between FCtox and CDC

In the early stages of the FCtox method development process a limited number of CDC versus FCtox comparisons were performed in order to address the assay sensitivity. No clear evidence of higher sensitivity for FCtox assay compared to CDC was observed (unpublished data). Here we re-evaluated this comparison using improved matched FCtox and CDC protocols. For instance, T and B cells used in CDC were here isolated by negative selection as compared to positive selection in the previous study and serum/complement incubation time were 30 + 30 min for both assays. Cells isolated from five separate (n = 5) donors were crossmatched against DSA positive sera at several dilutions (Table 1). The level of

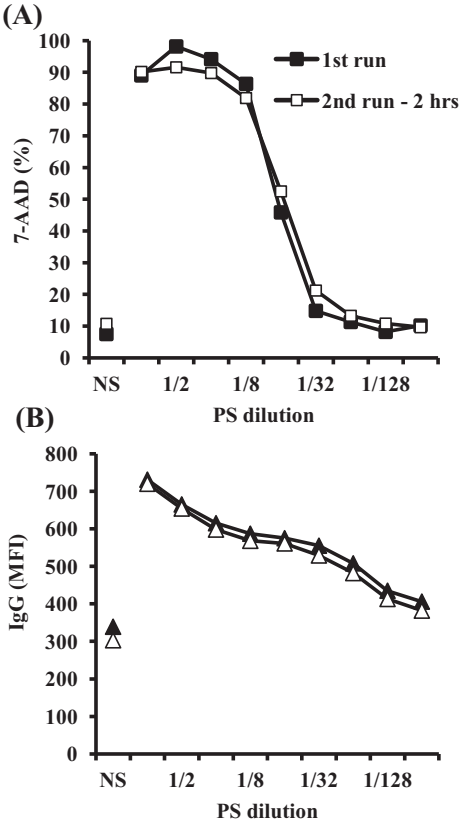

Fig. 5. Analysis of sample storage stability. Cytotoxicity (A) and IgG antibody binding (B) for B cells using negative serum (NS) and serially diluted (1/1 to 1/256) HLA antibody positive sera. Samples were acquired 10 min after completion of crossmatch ("1st run") and re-acquired after storage in refrigerator (+4 °C) for 2 h ("2nd run").

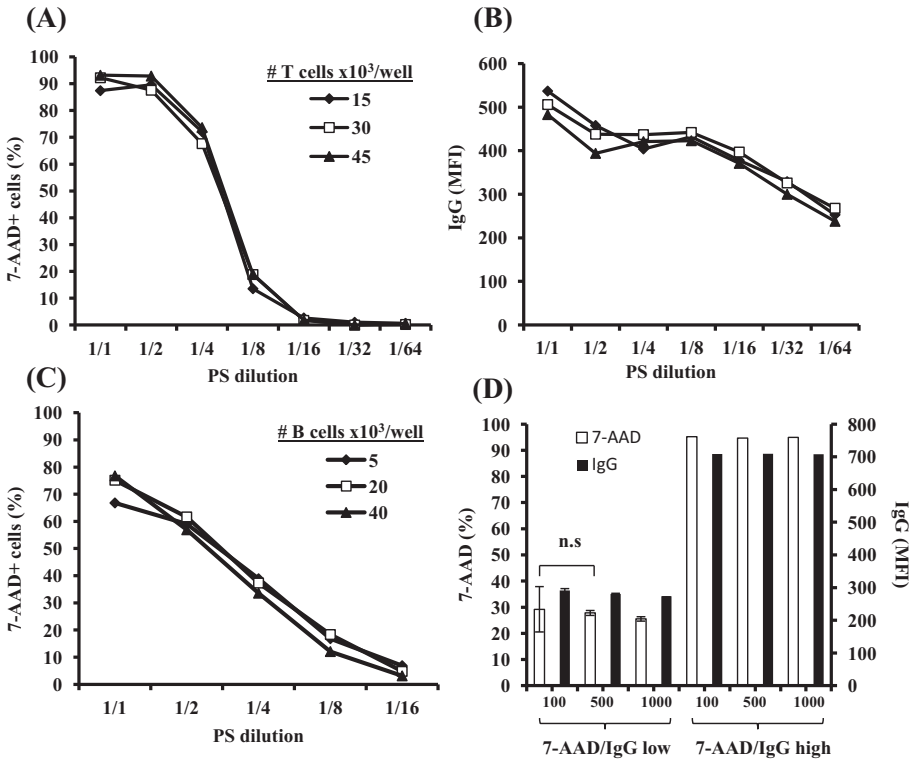

Fig. 6. Effect of different numbers of target cells per well and number of acquired events in FCtox crossmatch assay. Determination of T FCtox cytotoxicity (A) and IgG antibody binding (B) and B cell FCtox cytotoxicity (C) using different numbers of T and B cells/well. Crossmatches were performed on serially diluted HLA antibody positive sera (PS). The numbers of cells are indicated in graphs. (D) Assessment of FCtox cytotoxicity (unfilled bars) and IgG antibody binding (filled bars) upon acquisition of 100, 500 and 1000 FSC/SSC gated events from one 7-AAD/IgG "low" and one 7-AAD/IgG "high" sample. Data represents mean values (±SD) from five individual ($n = 5$) sample acquisitions for each group (100, 500 and 1000 events).

sensitivity for CDC and FCtox was comparable in most crossmatches. However, some exceptions were observed as shown in crossmatch No. 1 and 5 (XM#1 and 5). The percentage of FCtox 7-AAD+ cells was as high as 50–60% at 1/2 and 1/32 serum dilution in XM#1 and XM#5, respectively but the CDC reactivity (as judge by visual scoring) was negative or close to cut-off in those samples. In some CDC assays the scoring correlated surprisingly poor with the percentage of lysed cells as determined by FCtox. In XM#1 the 1/1 to 1/16 dilution were scored as 4 to 8 in CDC whereas the level of lysis were consistently high (~95–98%) for FCtox assay. The sera used in crossmatch XM#3–5 were assayed for complement fixing DSA antibodies by C1q LabScreen. Interestingly, in XM#5 both the FCtox (76% lysis) and CDC (score 6) outcome were clearly positive despite that no C1q fixing DSA were detected in this serum.

3.5. Deceased donor FCtox and CDC crossmatching

Deceased donors represent an important source of available donors in our cohort. It was therefore of importance to validate the FCtox assay using target cells isolated from these donors. Crossmatches for deceased donors were performed with DSA IgG negative and positive sera from kidney patients on the waiting list. In total seventy-three ($n = 73$) serum/cell combinations for both T and B cells were performed (Table 2). Approximately 60% of the crossmatches with DSA IgG+ sera induced cytotoxicity. In contrast, less than 10% with DSA IgG− sera induced cytotoxicity in the assay. DSA IgG+ sera resulted in IgG binding in 97% and 86% of the crossmatches for T and B cells, respectively. Thirty percent DSA IgG−/B FCtox IgG+ crossmatches were observed which is higher than previously seen for B cells isolated from live donors (15%) [14]. Higher frequencies of DSA IgG−/CDC+ compared to DSA IgG−/FCtox+ were observed for both T (18% vs 0%, $p = 0.002$) and B cells (23% vs 8%, $p = 0.08$). Side-by-side comparison of FCtox versus CDC in deceased donors showed a relatively large and significant proportion of T CDC+/T FCtox 7-AAD− (25%, $p < 0.0001$) and B CDC+/B FCtox 7-AAD− (18%, $p < 0.002$) crossmatches (Table 3). The T cell CDC reactivity for those eighteen ($n = 18$) T CDC+/T FCtox−crossmatches were score 2 in 11 cases ($n = 11$), score 4 ($n = 5$), score 6 ($n = 1$), and score 8 in one ($n = 1$) crossmatch. The B cell CDC reactivity for the B CDC+/B FCtox−crossmatches ($n = 13$) were score 2 in 10 cases ($n = 10$), score 4 ($n = 2$) andscore 6 ($n = 1$) crossmatch. In all but one of those CDC+/FCtox−crossmatches no DSA IgG and/or C1q class I antibodies were detected (data not shown).

4. Discussion

Our laboratory currently performs both CDC and flow crossmatching on live and deceased donors prior to transplantation. These assays are performed either in parallel for live donors or in sequence for deceased donors. In particular the CDC assay includes a large number of time-consuming and laborious manual steps such as preparation of plates, visual read-out/scoring and data registration which is not practical, efficient or objective enough for the HLA laboratory of today. Another drawback is the lengthy turnaround-time for the sequential crossmatch procedure. In an attempt to speed up this crossmatching process and circumvent some of the problems associated with conventional CDC we developed a flow cytometry based crossmatch that simultaneously measure cytotoxicity and antibody binding [14]. Here we further validated and improved this assay in order to fulfill routine HLA crossmatch assay requirements such as for instance reproducibility, assay robustness and stability. We demonstrate that the FCtox assay show excellent inter-operator, inter-instrument and

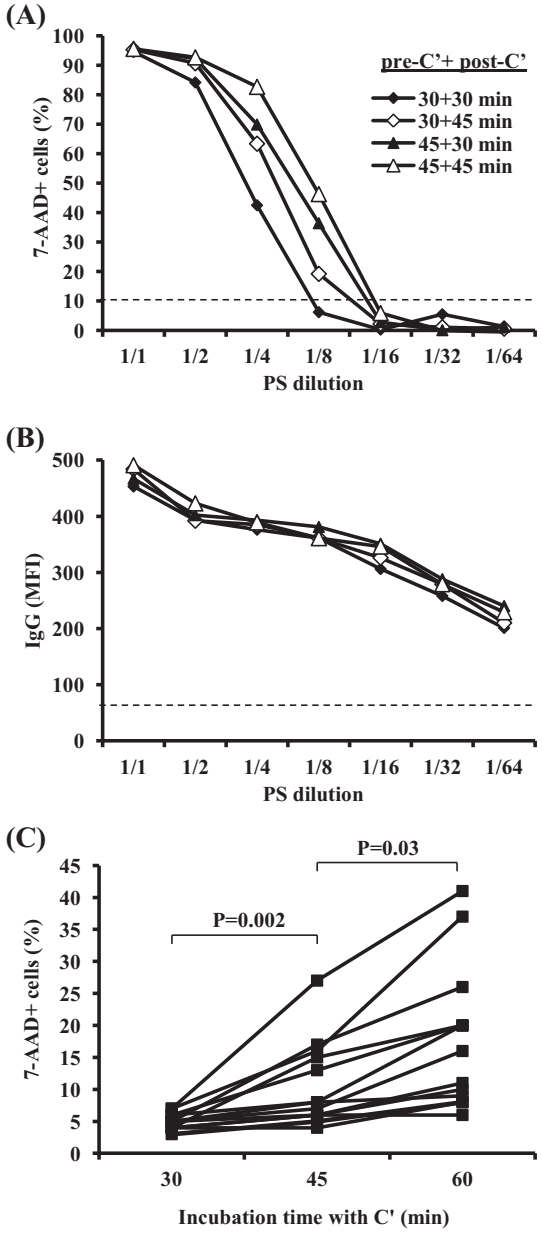

Fig. 7. Evaluation of serum and complement incubation times on cytotoxicity and IgG antibody binding. (A) T cell FCtox cytotoxicity and (B) T cell IgG antibody binding were determined with indicated serum (i.e. pre-C') and complement (i.e. post-C') incubation times. Crossmatches were done on undiluted (1/1) and serially diluted HLA antibody positive sera at indicated dilutions. The cut-off for 7-AAD (>10%) and IgG binding (>80 MFI) are indicated in the graphs with a dashed line. (C) Frequencies of B cell FCtox 7-AAD+ cells with negative serum using different complement incubation times (30, 45 and 60 min). Data from individual crossmatches (n = 13) are shown. Four different donors (n = 4) and five (n = 5) batches of negative sera were tested.

intra-assay reproducibility. These properties are of great importance in order to reach intra- and inter-laboratory result consistencies and harmonization. Another important finding was that sample storage prior to flow cytometry acquisition did not affect assay performance. The assay will thus withstand unexpected time delays prior to flow cytometry acquisition that may occur in the daily work. We also show that there is assay flexibility in terms of number of donor target cells/well and number of events that need to be acquired in order to obtain reproducible results. Conventional CDC assays normally require around 2000–3000 target cells/well. Occasionally we perform crossmatching for deceased donors on a large panel of patient sera (>60) and in those situations the number of B cells is a limiting factor. Since the amount of blood we receive from the donor is limited, it was of importance to determine how many B cells/well that were required to perform the FCtox assay. We show that even as low as 5000 cells/well is sufficient to obtain reliable results within reasonable time. Crossmatches performed with 5000 target cells/well resulted in acquisition time/sample around 30–40 s (500 events collected/sample) using the Cytomics FC500 instrument (data not shown). The FCtox assay is easy-to-use and time-to-result with the protocol described here did not exceed 3.5 h. More recently we have been able to reduce the turn-around-time even further by reducing the wash-time (1 min x2) and the secondary IgG incubation time (10 min RT) step (unpublished data). These and other improvements (e.g. using commercial PBMC collection devices or isolation of target cells directly from whole blood) will enable generation of both cytotoxicity and antibody binding results in less than 3 h. This reduced assay time opens up for an option to extend the serum and/or complement incubation times in order to increase sensitivity. Throughout this study we generally used incubation times of 30 min for both serum and complement which was sufficient to obtain specific HLA antibody dependent cell lysis and IgG binding. In case of a situation where low levels of complement fixing DSA are present an extended serum and complement incubation time (>30 min) may thus be required if only the cytotoxicity parameter of FCtox is applied. For example, the FCtox lysis in XM#3 (Table 1) at 1/4 dilution (19%) could be increased to 41% if 45 min instead of 30 min serum incubation time was used (data not shown). Notably, IgG binding above cut-off was repeatedly observed even at high serum dilutions and serum incubation time as short as 15 min (data not shown). Furthermore, we here show that increased complement time, above 45 min, occasionally resulted in unacceptable high levels of background cytotoxicity for B cells. This latter finding is particularly relevant in situations when the quality of donor blood is reduced; e.g. when analyzing PBMC from deceased donors or old blood samples.

In our initial evaluation comparable results were obtained between the FCtox assay and the conventional CDC and FCXM assays using blood from live donors [14]. Here we found that with deceased donors a somewhat lower degree of correlation between FCtox and CDC assays (Table 3). There was a 75% and 80% agreement between the results obtained by FCtox and CDC for T and B cells respectively, which is somewhat lower than previously observed with live donor cells (~90%) [14]. Importantly, FCtox resulted in significantly fewer "false positive" DSA IgG−/FCtox 7-AAD+ crossmatches compared to CDC. Furthermore, side-by-side comparison between FCtox 7-AAD and CDC shows a relatively large proportion of FCtox−/CDC+ crossmatches. In most of these disparate crossmatches no DSA IgG, IgM or C1q antibodies could be detected. In other words the FCtox assay is less likely to induce "false positive" reactivity in particular for deceased donor crossmatching. Antibodies to non-MHC or autoantigens are known causes of "false positive" crossmatch reactions [15]. However, we do not have any data indicating that these reactions are less likely to occur in FCtox. We believe instead that the design of FCtox assay (e.g. bead-free target cells, multiple washing steps and objective read-out) may reduce the number of nonspecific reactions. A limited number CDC and FCtox assays were performed in parallel using T and B cells isolated by negative selection and with same serum/complement incubation times (Table 1). The purpose of these experiments was to examine assay sensitivity for CDC vs. FCtox. Overall the assays exhibit similar sensitivity but occasionally higher levels of cytotoxicity were observed for the FCtox assay (e.g., XM#1 and 5).

Conventional CDC and flow cytometry crossmatching are widely used procedures in HLA laboratories. However, in recent years, prediction of patient/donor compatibility by virtual crossmatch

Table 1
Side-by-side comparison of cytotoxic reactivity for FCtox, CDC and C1q Labscreen.

Dilution[a]	XM#1		XM#2		XM#3			XM#4			XM#5		
	T 7AAD (%)[b]	T CDC score[c]	T 7AAD (%)[b]	T CDC score[c]	T 7AAD (%)[b]	T CDC score[c]	C1q MFI[d]	T 7AAD (%)[b]	T CDC score[c]	C1q MFI[d]	T 7AAD (%)[b]	T CDC score[c]	C1q MFI[d]
1/1	98	6	97	8	87	8	23889	98	8	20607	76	6	0
1/2	98	6	95	8	63	8	24302	99	8	23455	63	2	0
1/4	98	4	82	4	19	4	21486	98	8	23840	28	0	0
1/8	98	8	14	0	3	0	4340	99	8	22989	9	0	0
1/16	95	4	1	0	0	0	211	92	8	19993	3	0	0
1/32	57	0	0	0	0	0	0	54	4	5077	1	0	0
1/64	7	0	0	0	0	0	0	16	0	0	1	0	0

[a] HLA antibody positive sera used undiluted (1/1) and at indicated dilutions.
[b] Percentage of lysis above background (negative serum). Cut-off for positive reaction: >10%.
[c] CDC scoring: 0–10%: 0, 10–25%: 2, 25–50%: 4, 50–75%: 6, >75%: 8. Score 2 and above is considered positive.
[d] Mean fluorescence intensity (MFI) of DSA class I C1q fixing antibody. Cut-off for positive reaction: >1000 MFI.

Table 2
Deceased donor FCtox and CDC crossmatches against sera with and without donor-specific antibodies.

DSA IgG	T FCtox 7AAD[a]		T FCtox IgG[a]		DSA IgG	T CDC	
	Positive	Negative	Positive	Negative		Positive	Negative
Positive (n = 30)[b]	19/30 (63%)	11/30 (37%)	29/30 (97%)	1/30 (3%)	Positive (n = 60)[b]	29/60 (48%)	31/60 (52%)
Negative (n = 43)	0/43 (0%)	43/43 (100%)	3/43 (7%)	40/43 (93%)	Negative (n = 113)	20/113 (18%)	93/113 (82%)
	B FCtox 7AAD		B FCtox IgG		DSA IgG	B CDC	
DSA IgG	Positive	Negative	Positive	Negative		Positive	Negative
Positive (n = 36)[c]	22/36 (61%)	14/36 (39%)	31/36 (86%)	5/36 (14%)	Positive (n = 76)[c]	29/76 (38%)	47/76 (62%)
Negative (n = 37)	3/37 (8%)	34/37 (92%)	11/37 (30%)	26/37 (70%)	Negative (n = 97)	22/97 (23%)	75/97 (77%)

[a] The frequencies of T and B cell FCtox+ and FCtox–crossmatches against DSA IgG+ and IgG− sera.
[b] HLA class I specific antibodies.
[c] HLA class I and/or class II specific antibodies.

Table 3
Side-by-side comparison of deceased donor crossmatch results for FCtox and CDC.

T CDC[a]	T FCtox 7AAD[b]	
	Positive	Negative
Positive	19/73 (26%)	18/73 (25%)[c]
Negative	0/73 (0%)	36/73 (49%)
B CDC[a]	B FCtox 7AAD[b]	
	Positive	Negative
Positive	28/73 (38%)	13/73 (18%)[d]
Negative	1/73 (1%)	31/73 (42%)

[a] Complement-dependent cytotoxicity (CDC) assay was performed with whole blood isolated T and B cells.
[b] Crossmatch outcome for CDC *vs* FCtox.
[c] CDC score 2 (n = 11), score 4 (n = 5), score 6 (n = 1), score 8 (n = 1).
[d] CDC score 2 (n = 10), score 4 (n = 2), score 6 (n = 1).

(vXM) without the necessity of fresh donor cells has become a popular and effective tool in organ allocation (reviewed in [16]). vXM prediction is made possible by complete HLA typing of the donor and the determination of DSA by SAB technologies (LSA-IgG and LSA-C1q) [17,18]. Although the vXM methodology has several advantages to the conventional crossmatching, there are still situations where regular cell-based crossmatches are informative or even required. In order to adequately employ the vXM strategy, high resolution HLA typing (A, B, C, DR, DQ and DP) and careful analysis of DSA specificities are required. It has been argued that the sensitivity of IgG SAB assays is actually too high and potentially hinders the patient to obtain a graft [19,20]. The recently developed C1q assay could be a better tool for prediction of acute antibody mediated rejection since it only detects complement fixing antibodies [18,21]. Notably, we observed that the mean fluorescence intensity (MFI) levels of DSA C1q fixing antibodies do not always correlate with level of lysis as determined by FCtox (or CDC) which is in line with our previous observations [14]. Furthermore, determinations of HLA specific antibodies by single antigen beads (SAB) assays are sensitive to the prozone phenomenon caused by serum complement or IgM antibody [22,23]. If not reverted by treatment of serum (e.g. with EDTA or DTT) potentially harmful donor-specific antibodies will remain undiscovered and result in erroneous acceptance of patients for transplantation. Importantly, the cell-based complement dependent cytotoxicity (CDC) and FCXM assays are not influenced by the prozone effect [24]. We therefore advocate cell-based assays and believe that if improved, as described for FCtox, they will be beneficial for the transplantation outcome. The conventional CDC assay is based on microscope aided visual estimation of non-viable cells and is hence considered a relatively subjective assay, prone to operator induced errors. Experiments performed, here and previously, show that the FCtox assay is generally very reproducible within and between experiments, operators and instruments [14]. These are important findings which may enable future assay standardization and better harmonization between laboratories. Another advantage with the FCtox approach described here is that the 96-well plate format allows processing and analysis of large number of samples in a short time. This format paves the way for automated sample pipetting and washing procedures which will contribute to less operator induced errors and more reliable outcomes. Post-analytical errors such as manual result registration can easily be avoided by direct automatic transfer of data into the laboratory information system. The implementation of this assay may simplify and improve the day-to-day work in clinical transplantation. A multicenter evaluation of FCtox would clearly be an important step towards future implementation.

Conflicts of interest

The authors declare that they have no conflict of interest.

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