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ORIGINAL ARTICLE

A new flow cytometry assay identifies recipient IgG subtype antibodies binding donor cells: increasing donor availability for highly sensitised patients

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Abstract

Objectives. There are four immunoglobulin (IgG) subtypes that have varying complement-activating ability: strong (IgG3 and IgG1) and weak (IgG2 and IgG4). The standard flow cytometric crossmatch (FCM) assay does not distinguish between the various subtypes of the IgG molecule. This study outlines the development and use of a novel cell-based IgG subtype-specific FCM assay that is able to detect the presence of and quantitate the IgG subtypes bound to donor cells. **Methods.** A six-colour lyophilised reagent was designed that specifically detects the four IgG subtypes, as well as distinguishes between T cells and B cells in the lymphocyte population. To test the efficacy of this reagent, a retrospective evaluation of a group of highly sensitised patients awaiting heart and kidney transplant was carried out, who, because of positive standard FCM results, had been deemed incompatible with numerous prior potential donors. **Results.** Observations in this study demonstrate that the positive standard FCM results were mainly because of the presence of noncomplement-activating IgG2 or IgG4 antibodies. The results were supported by the absence of C3d-binding donor-specific antibodies (DSA) and a negative complement-dependent cytotoxicity crossmatch (CDC). **Conclusion.** Preliminary data presented in this study demonstrate the reliability of the novel IgG subtype assay to detect the presence of pretransplant, complement-activating antibodies bound to donor cells. The knowledge gained from the IgG subtype assay and the C3d-binding specificities of DSAs provides improved identification of donor suitability in pretransplant patients, potentially increasing the number of transplants.

Keywords: complement activation, flow crossmatch, IgG subtypes, sensitised patients, transplant

INTRODUCTION

Preformed antibodies to the donor's human leukocyte antigens (HLA) are characterised as donor-specific antibodies (DSA).[1] These antibodies can be directed towards HLA Class I antigens and/or Class II antigens of the donor.[2] The majority of DSAs detected in transplantation belong to the immunoglobulin G (IgG) or immunoglobulin M (IgM) class. Within the IgG molecule, there are four subtypes (isotypes): IgG1, IgG2, IgG3 and IgG4.[3,4] IgG subtypes differ in their relative abundance in human serum: IgG1 60%, IgG2 32%, IgG3 4% and IgG4 4%.[5] These subtypes bind complement with varying affinities.[6] For example, IgG1 and IgG3 are complement activating, whereas IgG2 and IgG4 are noncomplement-activating antibodies.[3] DSAs can also occur *de novo*. These *de novo* DSAs develop post-transplant and can lead to antibody-mediated rejection (AMR) if they are complement-activating IgG subtypes.

Currently, the complement-dependent cytotoxicity crossmatch (CDC) assay is the most widely used cell-based method to determine the presence of complement-activating anti-HLA antibodies.[7–9] This assay has relatively low sensitivity and resolution in determining the presence of complement-activating antibodies and can lead to false-negative results. Conversely, the highly sensitive, cell-based standard FCM assay relies on the level of fluorescent-tagged secondary pan-IgG antibodies that bind to and detect donor cell bound primary IgG antibodies.[10] This assay is not able to distinguish between complement-activating and noncomplement-activating IgG antibodies.[11] Identification of complement-activating anti-HLA antibodies in patient sera using a modification of the bead-based Luminex single-antigen bead (SAB) technology has been used to detect C1q, C3d or C4d products of complement activation. However, bead-based assays do not mirror the actual anti-HLA antibodies in patient sera that bind donor cells. Thus, there is a need to develop a comprehensive cell-based assay that combines the sensitivity of the standard FCM assay and the complement-activating ability of the CDC assay.

Developing assays having the ability to identify IgG subtypes would help quantify the level of noncomplement and complement-activating antibodies in sensitised patient sera. These levels could be provided during the transplant donor selection when there is a positive FCM result with a negative CDC result. This might allow prediction of hyperacute or accelerated AMR and graft loss, both pre- and post-transplantation. In addition, it has been proposed that the downstream complement cleavage product, C3d, is a better predictor of complement activation and graft loss through AMR in heart[12] and kidney[13] transplant recipients.

This study is based on the hypothesis that detecting the complement-binding ability of DSAs in highly sensitised recipients is possible by identifying the IgG subtypes using this novel cell-based assay.

RESULTS

Binding specificity of IgG subtypes lyophilised reagent and control beads

The lyophilised mixture was able to differentially identify between T and B cells. As shown in Figure 1, CD3-PE/Cy7 antibody in the lyophilised mixture specifically identified T cells in lymphocyte population, whereas B cells were specifically identified as a separate population by the CD19-APC/Fire™ 750 antibody in the same lyophilised mixture. No other antibodies in the lyophilised mixture showed any cross-reactivity with T or B cells in the lymphocyte population. Figure 1 is representative of three independent experiments. A random recipient serum/donor pair was used to demonstrate the gating strategy of lymphocytes and T and B cells, as well as the channel shift for each IgG subtype (Supplementary figure 1).

The anti-human IgG subtypes showed high degree of specificity to their primary targets and demonstrated the absence of cross-reactivity. Negative control beads were used to identify the beads on the flow cytometer by gating them on the histogram plot (Figure 2a). As seen in Figure 2b, a representation of three independent experiments, single positive peaks on the flow cytometric analysis were observed showing that each anti-IgG subtype antibody from the lyophilised mixture bound only to its corresponding primary IgG subtype antibody-coated polystyrene micro-bead. There was no cross-reactivity of the antibodies in the lyophilised mixture with any other primary IgG subtype-coated beads. This suggests that the customised

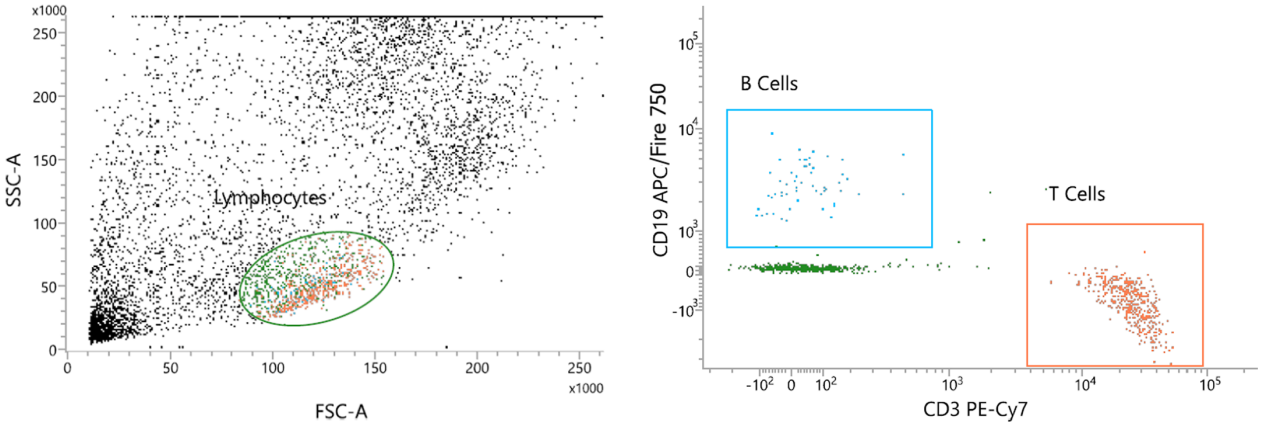

Figure 1. Human peripheral blood leukocytes were stained with six-colour lyophilised mixture. The dot plot was gated on lymphocytes. There was a clear differentiation of T cells (CD3) from B cells (CD19) in the dot plot. The T cells were specifically labelled with CD3-PE/Cy7, and the B cells were specifically labelled with CD19- APC/Fire™ 750 that were included in the six-colour lyophilised mixture. The figure is representative of three independent experiments.

lyophilised mixture was specific in recognising the IgG subtype antibody bound to the corresponding bead.

Validation of the IgG subtype assay

Identification of IgG subtypes in patient sera would serve as an important early indicator for the presence of complement-activating IgG1 and/or IgG3 antibodies and prediction of antibody-mediated rejection. Validation of the IgG subtype assay was carried out using sera samples from 50 patients and PBMC cells isolated from their respective donor blood. The standard CDC assay that determines the presence or absence of complement-activating antibodies in patient sera was also carried out using the same samples to correlate the findings from the IgG subtype assay.

A 94% positive concordance (47 of 50) was observed between the results from the standard CDC assay and IgG subtype assay in a majority of the cases in this study cohort. These include eight cases (16%) that were 'True Positive' as they were positive by the CDC assay and were also positive for the detection of complement-activating IgG1 and/or IgG3 antibodies in the IgG subtype assay. Also, there were 39 cases (78%) that were 'True Negative' by both CDC and IgG subtypes assays (detection of non-complement-activating IgG2 and/or IgG4 antibodies). Three cases (6%) were observed to be negative by the CDC assay, but the IgG subtype assay detected the presence of complement-activating IgG1 and/or IgG3 antibodies. These were termed as 'False Positive' for the IgG subtype results. There were 0 'False Negative' cases that were positive for CDC but negative for the IgG subtype assay. All these observations suggest that the IgG subtype assay has a sensitivity of 100%, specificity of 92.86% and F1 score (measure of a test's accuracy) of 0.88 to accurately predict the presence of complement-activating IgG1 and/or IgG3 antibodies.[14] Furthermore, the IgG subtype assay has a positive predictive value (*P*-value) of 72.73% and a negative *P*-value of 100% to accurately predict the presence or absence of complement-activating IgG subtypes.

Although both CDC and IgG subtype assays determine the presence of complement-activating antibodies in patient sera, the superiority of the IgG subtype assay lies in the fact that it is highly sensitive than the CDC assay. In addition, the IgG subtype assay has the ability to identify the presence of IgG1 and/or IgG3 subtypes that are responsible for the activation of the complement cascade leading to AMR.

IgG subtypes assay facilitates successful heart transplantation in highly sensitised patients

The study cohort consisted of seven highly sensitised patients (Table 1) listed for heart transplantation having an average pretransplant peak calculated panel reactive antibody (cPRA) of

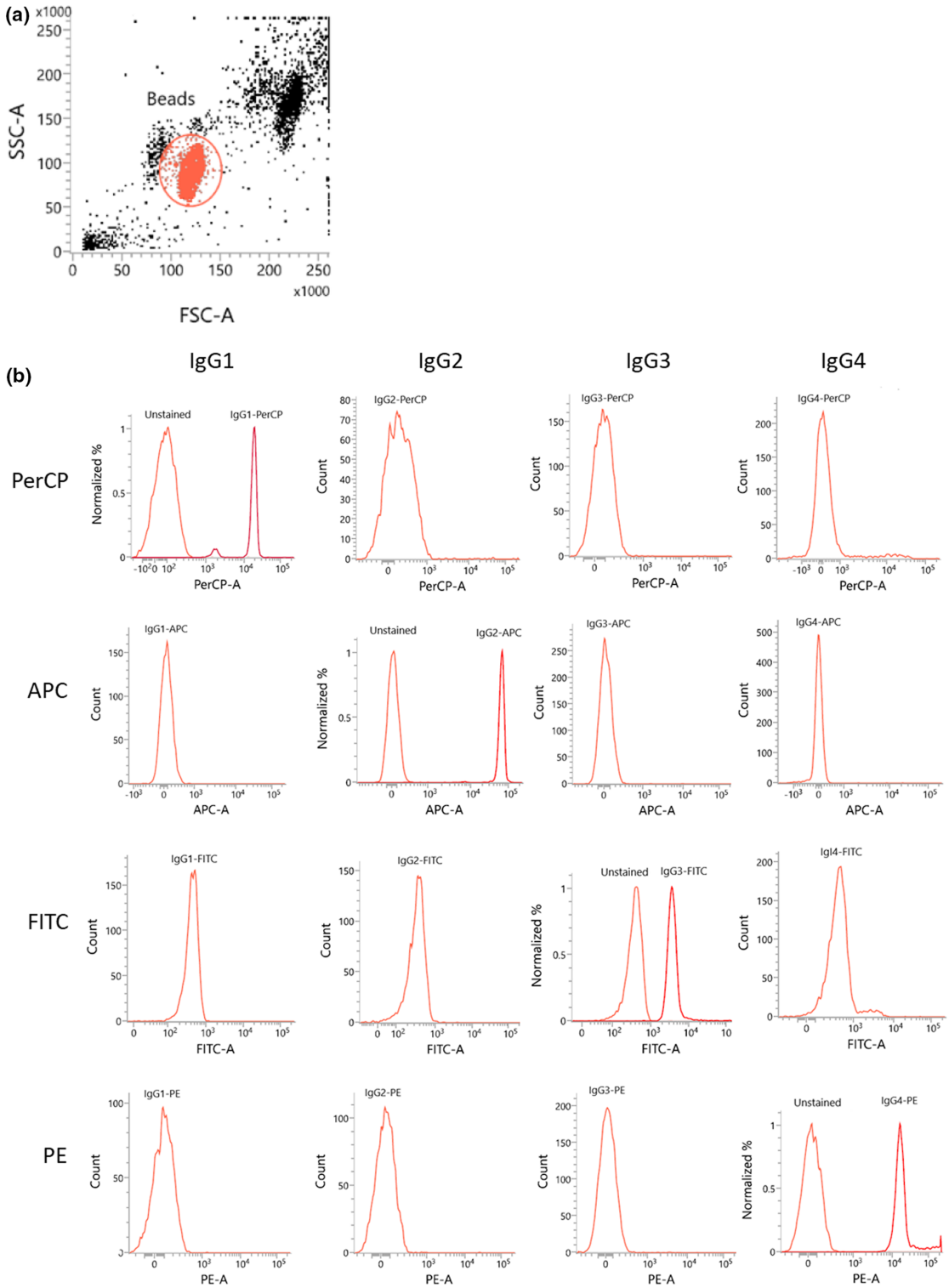

Figure 2. (a) Control polystyrene microbeads that were coated individually with IgG1, IgG2, IgG3 and IgG4 antibodies were incubated with the hydrated 6-colour lyophilised mixture reagent. The beads were washed and analysed by flow cytometry. Histograms were gated on the polystyrene microbeads. A representative figure of three independent experiments is shown. **(b)** Control beads that were conjugated with primary IgG subtype antibodies as well as negative control beads were incubated with hydrated six-colour lyophilised mixture reagent and analysed by the flow cytometry. Specific staining of each individual IgG subtype-coated bead was observed with no cross-reactivity with any other IgG subtype antibody from the six-colour lyophilised mixture reagent. A representative figure of three independent experiments is shown.

89.12%. These patients were deemed incompatible with an average of 16 donors because of a positive standard FCM result. There were six patients that displayed both Class I and Class II DSAs to their respective donors. The FCM results were positive in these patients.

Recipients #7, #1 and #5 had strong B- and T-cell FCM results, but negative CDC results suggesting the absence of complement-activating antibodies in the recipient serum. Recipients #1 and #5 had Class I DSAs that were negative for binding C3d complement, suggesting the absence of complement-activating DSA. None of the recipients had a predominance of any of the complement-activating or noncomplement-activating IgG subtype antibodies which supports the CDC results. Our observations suggest a successful heart transplant for these recipients even though they had a positive FCM result.

Recipient #6 had positive FCM and CDC results suggesting the presence of complement-activating antibodies in the pretransplant recipient serum that were binding donor cells. Results from our IgG subtype assay contradicted these observations as we did not see an increase in the levels of either complement or noncomplement-activating IgG subtype antibodies in the recipient serum. Recipient #6 had Class II DSAs that were negative for binding C3d complement, suggesting that they were noncomplement-binding antibodies. As a result of the increased sensitivity of our IgG subtype assay and the absence of complement-activating IgG1 or IgG3 subtype antibodies, our observations suggest that recipient #6 could successfully receive heart transplant from the potential donor even in the presence of a positive FCM and CDC results.

We observed that the positive FCM result for recipient #2 was because of an increase in the levels of the noncomplement-activating IgG2 subtype antibodies. The negative CDC result, as well as Class I DSA that was negative for binding C3d complement, supports our observations for the absence of complement-activating antibodies in the recipient serum.

Recipient #3 had a positive standard FCM result; however, observations from our IgG subtype assay suggest the predominance of noncomplement-activating IgG2 antibodies could be causing the positive standard FCM result. The negative CDC result supports our IgG subtype observations for the absence of complement-activating antibodies. Recipient #3 had Class II DSAs that were analysed for their ability to bind C3d complement. We observed that one of the DSAs, DRB3, was positive for C3d binding. Although the C3d assay results contradict IgG subtypes and CDC assay results, the clinical post-transplant outcomes for recipient #3 were very favorable and suggested no AMR of the transplanted heart. As mentioned in the Validation section of the manuscript, our IgG subtypes assay has a specificity of 92.86% and *P*-value of 72.73%. This aligns with the nonuniformity of our IgG subtypes result with comparable C3d assay result, since not all comparable diagnostic testing platforms always correlate with 100% accuracy.

Recipient #4 had a strong positive standard FCM result with the potential donor. When we performed our IgG subtype assay, we observed that the positive standard FCM was because of increased levels of noncomplement-activating IgG2 (T cells) and IgG4 (B cells) subtype antibodies in the pretransplant recipient serum. Recipient #4 also had both Class I and Class II DSAs. Of these, only one of the Class I DSAs, A24, was weakly positive for binding C3d complement. Overall, our observations suggest that #4 could receive the heart transplant in the presence of a positive standard FCM and weak Class I C3d binding DSA based on the prevalence of noncomplement-activating antibodies as shown by our IgG subtype assay. As mentioned earlier, our IgG subtypes assay and comparable C3d assay do not always correlate because of the different testing methodologies. Our observations are supported by the negative CDC assay result suggesting the absence of complement-activating antibodies that would not lead to rejection of the transplanted organ because of AMR.

Post-transplant clinical outcomes for all heart transplant recipients in our study were very favorable, supporting our pretransplant IgG subtypes assay predictions. There was no primary graft dysfunction (PGD) in any of the heart transplant recipients, and the 30-day and 90-day graft survival parameters were favorable. The recipients were maintained on prescribed minimal immunosuppression post-transplant. There was no C4D staining of the endomyocardial biopsy (EMB) suggesting absence of AMR. Furthermore, the pathologic antibody-mediated rejection (pAMR) gradation as per the International Society for Heart and Lung Transplantation (ISHLT) guidelines was pAMR0 suggesting negative histologic and immunologic findings at 2 weeks post-transplant and, after 2 weeks post-transplant.

Table 1. Highly sensitised heart transplant patients having HLA Class I and Class II DSAs

				IgG subtypes							
	FCM			B cells				T cells			
Recipient #	B cell	T cell	CDC	IgG 1	IgG 2	IgG 3	IgG 4	IgG 1	IgG 2	IgG 3	IgG 4
1	POS	POS	NEG	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
2	NEG	POS	NEG	< 2	< 2	< 2	< 2	< 2	4.5	< 2	< 2
3	POS	NEG	NEG	< 2	< 2	< 2	< 2	< 2	3	< 2	< 2
4	POS	POS	NEG	< 2	< 2	< 2	2.5	< 2	3.1	< 2	< 2
5	POS	**POS**	NEG	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
6	POS	NEG	POS	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
7	POS	POS	NEG	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2

						Clinical outcomes					
Recipient #	DSA	Specificity	MFI	C3d	C4D on Bx	Primary graft dysfunction (PGD)	Survival 30 days post TxP	Survival 90 days post TxP	Induction	Bx findings – 2 weeks post-TxP	Bx findings – after 2 weeks post-TxP
1	B44	B*44:03	11,715	NEG	None	No	Yes	Yes	ATG	pAMR 0	pAMR2
2	A2	A*02:03	3,343	NEG	None	No	Yes	Yes		pAMR 0	pAMR 0
3	DR13	DRB1*13:03	16,620	POS	None	No	Yes	Yes	ATG	pAMR 0	pAMR 1
	DRB3	DRB3*03:01	8,185	NEG							
4	A24	A*24:03	15,243	Weak POS	Mild C4D	No	Yes	Yes	None	pAMR 0	pAMR 0
	B58	B*58:01	11,471	NEG							
	DRB4	DRB1*04:01	4,140	NEG							
5	B72	B*15:03	1,446	NEG	None	No	Yes	Yes	IVIg and plasmapheresis, and rituximab	pAMR2	Intermittent pAMR2
6	DRB3	DRB3*02:02	2,921	NEG	None	No	Yes	Yes	None	pAMR 0	pAMR 0
	DQ7	DQB1*03:01	3,988	NEG							
7	–	–	–	NEG	None	No	Yes	Yes	IVIg and Steroids	pAMR 0	pAMR 0

ATG, antithymocyte globulin; IVIG, intravenous immunoglobulin; pAMR, pathologic antibody-mediated rejection.
Results of tests performed are listed. These tests include the standard FCM, CDC, IgG subtypes, C3d, and C4d assay. Donor cells were incubated with negative human serum to define the baseline of antibody binding. The same donor cells were simultaneously incubated with recipient serum in separate wells. The numbers in the table represent fold-change in the binding of IgG subtypes in recipient to donor cell surface over this baseline. Treatment of donor cells with negative human serum and recipient serum was made in duplicate wells. * < 2 – IgG subtype levels below twofold threshold cut-off and considered negative.

IgG subtypes assay facilitates successful kidney transplantation in highly sensitised patients

We evaluated 14 sensitised patients awaiting kidney transplantation. These patients were on the wait list for more than 3 years with an average cPRA of 76%. When a donor kidney was available for these patients, we performed a CDC assay to determine the presence of complement-activating antibodies in the patient serum that could bind the donor cells and induce cell lysis through the activation of the complement pathway. As seen in Table 2, CDC results were negative for all patients awaiting kidney transplant, suggesting these patients could safely receive the donor kidney. No CDC was performed for recipients #5 and #6.

Recipient #2 had no DSA, but a positive B-cell FCM. The IgG subtypes assay showed that the positivity of the FCM was because of a predominance of noncomplement-activating IgG2 antibodies binding to the donor cells. Recipient #2 also had a negative CDC supporting the IgG subtype results. Similarly, recipient #8 had positive B- and T-cell FCM; however, our IgG subtypes assay demonstrated a predominance of the noncomplement-binding IgG2 subtype for the T cells while there was no substantial increase in any of the IgG subtypes for B cells. Although recipient #8 had a Class I DSA, it was negative for binding C3d complement further supporting the

observations from our IgG subtype results. Our observations were supported by the negative CDC assay results suggesting an absence of complement-activating antibodies in the patient sera.

Recipients #4, #5, #12 and #14 had both negative FCM and CDC results suggesting the absence of complement-activating antibodies. Recipient #5 had a negative C3d binding Class II DSA supporting the absence of complement-activating antibodies. Our IgG subtypes assay supports these results as these recipients had increased levels of non-complement-activating IgG2 or IgG4 antibodies binding the donor cells. Recipients #3, #7, #9 and #10 had either a positive B- or T-cell FCM but negative CDC results suggesting the absence of complement-activating antibodies. Recipients #7 and #10 had Class II DSAs, but they were C3d negative suggesting that these DSAs were unable to bind complement. Our IgG subtype results showed no overall increase in any of the complement and noncomplement-activating antibodies in the pretransplant sera of these recipients suggesting that they were suitable to proceed with kidney transplantation.

Recipient #13 had a negative FCM result and a Class II DSA that was negative for binding C3d complement. Both recipients #13 and #1 had negative CDC results suggesting the absence of complement-activating antibodies. These results were supported by our IgG subtypes assay as there were no increased levels of any of the IgG subtypes for neither B nor T cells, suggesting that both these recipients could receive their respective donor kidneys and not experience rejection because of AMR post-transplant.

There were only two recipients whose IgG subtypes results showed the presence of complement-activating IgG3 antibodies. Recipient #6 had negative FCM and CDC results and no DSAs, suggesting suitability for transplant. However, our IgG subtypes assay showed an increase in complement-activating IgG3 levels suggesting a risk of rejection of the transplanted kidney because of AMR. Another recipient, #11, had a positive B- and T-cell FCM. Our IgG subtypes assay showed an increase in the levels of complement-activating IgG3 antibody binding to the donor cells. This suggests that the positive FCM for #11 was because of the presence of IgG3 antibodies, thus predicting rejection of the transplanted kidney because of AMR. Recipient #11 had both Class I and Class II DSAs that were negative for C3d binding suggesting the absence of complement-activating antibodies. These observations were in contrast to our observation with the IgG subtype assay. This discrepancy could be because of the difference between the less sensitive CDC assay and the highly sensitive IgG subtype assay that can detect the subtypes of low levels of IgG antibodies in the recipient sera.

The post-transplant clinical parameters obtained from a retrospective chart review showed that the creatinine levels remained stable for all the patients in this cohort. The recipients were maintained on standard immunosuppression regimens such as tacrolimus and mycophenolate mofetil (MMF) along with thymoglobulin induction. There was no evidence of AMR in any patient in this study group, further supporting the IgG subtype observations. Although three patients died 4–7 years post-transplant, this was not because of kidney failure as they all died with functioning kidney allografts. Overall, results of the IgG subtype assay correlated with the standard CDC results as well as the C3d assay results. As the IgG subtype assay is not yet part of the clinical testing menu, no clinical decisions were made for the patients based on the results of the IgG subtype assay.

A representative case is discussed that best describes the clinical usefulness of the IgG Subtype assay (Recipient #4). This 63-year-old African American male waited for a heart transplant for over 4 years. During this wait period, the patient had 58 positive virtual crossmatches and standard FCM results with potential donors. He received multiple transfusions, was hospitalised for infection and underwent left ventricular assist device implantation increasing his sensitisation. His peak cPRA was 100%. When the next potential donor was identified, a standard FCM was performed. The result was positive for both T and B cells as expected. The IgG subtype assay was subsequently carried out using the same serum sample and donor cells. Of the four IgG subtypes, only subtypes 2 and 4 were identified in the recipient's serum, bound to donor cells. Based on the results of the IgG subtype assay, a prediction of successful transplant could be made since only IgG subtypes 1 and 3 are known to be complement-binding. However, the clinical decision to transplant was based on a negative CDC result which is currently a standard practice in transplantation. After the patient received the

Table 2. Highly sensitised kidney transplant patients having HLA Class I and Class II DSAs

				IgG subtypes							
	FCM			B cells				T cells			
Recipient	B cell	T cell	CDC	IgG 1	IgG 2	IgG 3	IgG 4	IgG 1	IgG 2	IgG 3	IgG 4
1	–	–	NEG	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
2	POS	NEG	NEG	< 2	2.67	< 2	< 2	< 2	< 2	< 2	< 2
3	POS	NEG	NEG	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
4	NEG	NEG	NEG	< 2	< 2	< 2	< 2	< 2	< 2	< 2	4.08
5	NEG	NEG	–	< 2	< 2	< 2	< 2	< 2	7.33	< 2	< 2
6	NEG	NEG	–	< 2	< 2	2.13	< 2	< 2	2.5	< 2	< 2
7	NEG	POS	NEG	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
8	POS	POS	NEG	< 2	< 2	< 2	< 2	< 2	7.0	< 2	< 2
9	NEG	POS	NEG	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
10	POS	NEG	NEG	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
11	POS	POS	NEG	< 2	< 2	2.74	< 2	< 2	< 2	< 2	< 2
12	NEG	NEG	NEG	< 2	< 2	< 2	< 2	< 2	2.32	< 2	< 2
13	NEG	NEG	NEG	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
14	NEG	NEG	NEG	< 2	< 2	< 2	< 2	< 2	4.15	< 2	7.6

					Clinical outcomes									
Recipient	DSA	Specificity	MFI	C3d	Preemptive transplant	Nadir post-Txp. Cr.	Current Cr./date	Induction	Maint. immuno meds	Dialysis first week	Rejection episodes	Bx finding	C4D on Bx	Notes
1	–	–	–	–	No	1.54	1.69 on 11/27/2019	Thymo induction	Tacrolimus, myfortic	Yes	No	6/2017: no ACR or AMR, (–)C4d, mod IF/TA, ATN	Negative	–
2	–	–	–	–	Yes	1.15	1.33 on 1/18/2021	Thymo induction	Tacrolimus, mycophenolate mofetil	No	No	N/A	N/A	–
3	–	–	–	–	Yes	0.84	0.96 on 1/3/2020	Thymo induction	Tacrolimus, mycophenolate mofetil	No	No	N/A	N/A	–
4	–	–	–	–	No	0.39	0.42 on 7/31/2019	Thymo induction	Tacrolimus, myfortic	No	No	N/A	N/A	–
5	DR13	DRB1*13:01	1195	NEG	No	1.18	1.25 on 3/8/2021	Thymo induction	Tacrolimus, mycophenolate mofetil	No	No	N/A	N/A	–

(Continued)

Table 2. Continued.

					Clinical outcomes									
Recipient	DSA	Specificity	MFI	C3d	Preemptive transplant	Nadir post-Txp. Cr.	Current Cr./date	Induction	Maint. immuno meds	Dialysis first week	Rejection episodes	Bx finding	C4D on Bx	Notes
6	–	–	–	–	No	0.7	4.76 on 3/6/2021	Thymo induction	Tacrolimus, myfortic	No	No	2/2018: no evidence of AMR or ACR, C4d staining negative, approximately 30% GS and mod IF/TA; 12/22/2020: Banff 2A ACR, acute AMR, mod IF/TA	Negative	Patient with noncompliance prior to 12/2020 biopsy. Cr had been stable at 1.3 before noncompliance
7	DQB2	DQB1*02:01	1229	NEG	No	1.18	1.47 on 10/30/2018	Thymo induction	Tacrolimus, myfortic	No	Yes	7/2018: Banff 2A ACR, no AMR, C4d(−)	Negative	–
8	B45	B*45:01	1976	–	No	0.98	0.98 on 1/13/2021	Thymo induction	Tacrolimus, myfortic	No	No	N/A	N/A	–
9	–	–	–	–	No	0.87	0.87 on 7/30/2019	Thymo induction	Tacrolimus, myfortic	No	No	N/A	N/A	Death with functioning allograft 5/2020
10	DQB2			NEG	No	1.11	1.38 on 1/24/2021	Thymo induction and plasmapheresis	Tacrolimus, myfortic	No	No	10/2014: no ACR/AMR, C4d(−), mild IF/TA	Negative	Death with functioning allograft 1/2021
11	A1 B7 DR15 DR51	A*01:01 B*07:02 DRB1*15:01 DRB5*02:02	2995 3368 5224 12862	NEG NEG NEG NEG	No	0.82	1.00 on 10/29/2020	Thymo induction, eculizumab, plasmapheresis	Tacrolimus, myfortic	No	No	1/2015: no ACR/AMR, C4d(−), minimal IF/TA	Negative	–
12	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13	DR7	DRB1*07:01	2467	NEG	–	–	–	–	–	–	–	–	–	–
14	–	–	–	–	–	–	–	–	–	–	–	–	–	–

ACR, acute cellular rejection; AMR, antibody-mediated rejection; ATN, acute tubular necrosis; IF/TA, interstitial fibrosis and tubular atrophy.

Results of tests performed are listed. These tests include the standard FCM, CDC, IgG subtypes, C3d, and C4d assay. Donor cells were incubated with negative human serum to define the baseline of antibody binding. The same donor cells were simultaneously incubated with recipient serum in separate wells. The numbers in the table represent fold-change in binding of IgG subtypes in recipient to donor cell surface over this baseline. Treatment of donor cells with negative human serum and recipient serum was made in duplicate wells. * < 2 – IgG subtype levels below twofold threshold cut-off and considered negative.

heart transplant, he received induction therapy with basiliximab and protocol immunosuppression with prednisone, tacrolimus and mycophenolate. There was no primary graft dysfunction, nor graft failure during the first post-transplant year. Biopsy samples stained for C4D were negative suggesting no evidence of AMR. An echocardiogram performed 12 days post-transplant showed normal biventricular function. The patient is now 7 years post-transplant with continued good health and no evidence of rejection.

DISCUSSION

This study reports, for the first time, in patients awaiting heart and kidney transplant, the use of a cell-based IgG subtype assay that combines the high sensitivity of the standard FCM assay and the complement detecting specificity of the CDC assay. The unique lyophilised reagent of fluorescent-tagged secondary anti-IgG antibodies detect the subtypes of the IgG antibodies bound to the donor cells. These secondary antibodies bind with high specificity to their respective IgG subtypes and do not cross-react with other IgG subtypes on the donor cells. The IgG subtype assay has the potential of being a high-throughput testing modality that can detect all four IgG subtypes bound to the donor cells in a single reaction. This assay essentially performs four FCM tests in one test tube.

Non-cross-reactivity of the fluorescent-tagged antibodies in the lyophilised reagent that can be detected independently by flow cytometric analysis has been successfully demonstrated in this study. Validation of this reagent using donor PBMCs and patient sera shows that the IgG subtype assay is a sensitive and highly specific assay that can detect the presence of PBMC-bound IgG subtypes with high accuracy. Overall, the six-colour lyophilised reagent can identify IgG subtypes in patient sera that are bound to donor cells and predict the presence or absence of complement-activating antibodies. This assay has the potential to help in facilitating better management of patient care and increasing the number of successful transplants.

The IgG subtypes assay has been tested in a small group of seven heart transplant and 14 kidney transplant patients. Most of the patients in this study cohort had a positive standard FCM result against their respective potential organ donors, thus making them unsuited for transplantation based on current criteria. However, this study demonstrated that successful heart and kidney transplantation could be achieved in these patients having a positive FCM because of the presence of noncomplement-activating antibodies as observed from the IgG subtype results.

The downstream complement component, C3d, can bind DSAs and act as a marker for complement-activating antibodies.[15] In recipients having multiple DSAs, the C3d binding assay can detect which DSA specificities are C3d positive suggesting their ability to bind complement.[13] Observations in this study are in agreement with earlier reports of the usefulness of this assay to detect weak complement-activating DSAs, thus enabling better management of pretransplant desensitisation protocols.

There are reports showing some patients having DSAs exhibit normal graft function.[16,17] This suggests that there must be properties that distinguish intrinsically less pathological or nonpathological DSAs from the pathological DSAs. The IgG subclasses of antibodies that bind to the donor cells were evaluated using the in-house developed IgG subtype assay. Patients in this study group were deemed ineligible for transplant because of a positive standard FCM result, even though they had a negative CDC assay result. Rationale for focussing on pretransplant patients was to enable highly sensitised recipients to receive an organ transplant.

Studies involving the detection of IgG subtypes in post-transplant patients using Luminex® bead-based assays have shown that acute AMR is mainly driven by the increase in IgG3-DSA and associated with a greater risk of graft loss, whereas subclinical AMR was associated with an increase in IgG4-DSA.[4,18] Zeevi *et al*.[19] have demonstrated that persistent presence of post-transplant C1q-binding anti-HLA antibodies in sensitised heart transplant patients likely indicates the presence of complement-fixing IgG1 and/or IgG3 subclass antibodies associated with AMR. Furthermore, the detection of pretransplant complement-fixing antibodies has helped in reducing the incompatible donor pool for highly sensitised patients.[20] Incorporation of the C1q assay to support observations from the IgG subtype assay did not show reproducible results during in-house testing. Therefore, C1q assay was not included in this study, whereas the C3d

binding assay that had consistently shown reproducible results was used to support the IgG subtypes assay results in this study.

IgG subtypes detected using the bead-based assay does not reflect the actual antibodies that bind to the respective donor cells. Binding of anti-HLA antibodies in patient sera is donor-cell dependent. Variable complement-binding sensitivity of these antibodies gives rise to a situation where a patient could be compatible with one donor because of binding of noncomplement-activating antibodies, while being totally incompatible with another donor because of increase in binding of complement-activating antibodies.[21]

CONCLUSION

According to the United Network for Organ Sharing (UNOS), there are ~106,000 people in the United States waiting for a transplant. A significant subset of these are highly sensitised patients that routinely test positive for the standard FCM. The impact of the IgG subtype assay could be substantial, even if utilised only for highly sensitised patients. The IgG subtype assay may be helpful in cases of a positive flow crossmatch and may facilitate more transplants than currently performed. Further augmentation could be achieved by identifying the specificity of the C3d-positive DSAs. Integration of both these assays in the transplant testing regimen for patients awaiting solid organ transplant may aid in increasing donor availability for highly sensitised pretransplant patients.

METHODS

Study statements

This study was approved by the Institutional Review Board (2020-0.57, dated 4/28/2021) of Newark Beth Israel Medical Center, Newark, NJ, USA, and the Institutional Review Board (ID: 21-13) of the Saint Barnabas Medical Center, Livingston, NJ. This study abides by the Declaration of Helsinki principles. No animals were used in this study.

Preparation of six-colour IgG subtypes cocktail reagent

The proprietary secondary antibodies consist of a lyophilised reagent that recognises the various IgG subclasses. The six-colour lyophilised cocktail reagent was manufactured by BioLegend, San Diego, CA, USA. The lyophilised reagent consists of a combination of antibodies as follows: PE/Cy7 conjugated to anti-human CD3 antibody clone UCHT1 that binds to T cells and APC/Fire™ 750 conjugated to anti-human CD19 antibody clone HIB19 that binds to B cells. The combination of secondary antibodies used includes PerCP conjugated to anti-human IgG1 antibody clone 12G8G11, APC conjugated to anti-human IgG2 antibody clone HP6002, FITC conjugated to anti-human IgG3 antibody clone HP6047 and PE conjugated to anti-human IgG4 antibody clone HP6023 (Supplementary table 1a). The antibodies were purified by affinity chromatography, treated and formulated at optimal concentrations for multicolor flow cytometry. These antibodies were conjugated to their respective fluorochromes under optimal conditions. The formulation was performed in phosphate-buffered saline (PBS) solution at pH 7.2 and containing bovine serum albumin (BSA) as stabiliser. The six-colour lyophilised cocktail reagent was free of unconjugated fluorochromes and unconjugated antibodies. The reagent passed all quality testing by flow cytometry and was certified to use by BioLegend's QC testing under an ISO 13485:2016 certified quality management system (https://www.biolegend.com/qc).

Preparation of control beads

The lyophilised positive control reagent consists of standardised polystyrene microbeads conjugated to affinity chromatography-purified individual IgG subtype antibodies (IgG1, IgG2, IgG3 and IgG4) (Supplementary table 1b). The lyophilised negative beads consist of polystyrene microbeads that have no conjugated antibody on the surface. The formulation was performed in PBS solution at pH 7.2 containing BSA as stabiliser.

The lyophilised compensation control for T cells was prepared separately by conjugating PE/Cy7 to anti-human CD3 antibody (Figure 3a). The B-cell-lyophilised compensation control was prepared by conjugating APC/Fire™ 750 to anti-human CD19 antibody (Figure 3b). The formulation was performed in PBS solution at pH 7.2 containing BSA as stabiliser. Figure 3a and b are representative of three independent experiments.

Sample collection

Whole blood was collected from recipients per the routine standard of care in BD Vacutainer® Serum tubes containing no anticoagulant or preservative. Sera was isolated and stored at −20°C for the IgG subtypes and C3d analysis. Whole blood from donors for the respective recipients was collected in acid citrate dextrose (ACD) tubes and used for isolation of peripheral blood mononuclear cells (PBMCs).

HLA typing

DNA typing for HLA Class I (HLA-A, -B, -C) and Class II (HLA-DRB1, -DRB3/4/5, -DQB1, -DP) was carried out using LABType® SSO (One Lambda Inc., Canoga Park, CA, USA) based on the reverse sequence-specific oligonucleotide probe (rSSO) DNA typing method. Briefly, the target DNA

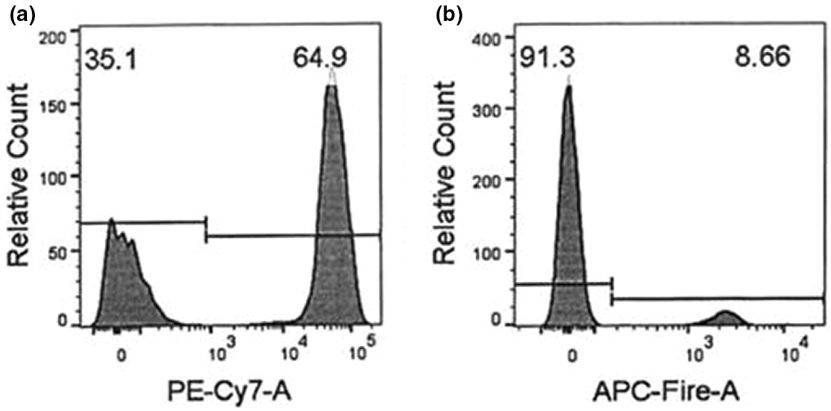

Figure 3. (a) Lyophilised compensation control antibody for T cells showing a positive peak on flow cytometric analysis. The negative peak is the isotype control antibody. **(b)** B cells are recognised by the lyophilised compensation control antibody showing a positive peak on flow cytometric analysis. The negative peak is the isotype control antibody. **a** and **b** are representative of three independent experiments.

was amplified by polymerase chain reaction (PCR) using locus-specific primers and then hybridised to complementary DNA probes that are chemically bound to fluorescently coded microspheres and detected in a single reaction mixture using the Luminex xMAP® Technology (Luminex, Austin, TX, USA).

Identification of HLA antibody specificities

All patients underwent screening for HLA-specific antibodies. Initial screening was performed to detect the presence or absence of HLA class I and/or class II antibodies using FlowPRA screen beads that are coated with purified HLA class I or class II antigens (One Lambda Inc.). Individuals with positive panel-reactive antibody (PRA) for HLA class I, II, or both were further evaluated using LABScreen single-antigen beads (SAB) assay for identifying the specificities of the HLA class I or class II antibodies (One Lambda Inc.). This assay detects antibodies to HLA A, B, Cw, DR, DQ and DP antigens at the allele level. Briefly, the recipient serum was incubated with the SAB beads that were coated with purified Class I and Class II HLA antigens. The beads were then washed and the bound antibodies were labelled with R-Phycoerythrin (PE)-conjugated goat anti-human IgG. The LABScan 100 flow analyser (Luminex) was used to detect the fluorescent emission of PE from each bead and assigned the HLA specificity.

Standard flow cytometric crossmatch

The presence of preformed DSA was detected using the highly sensitive standard FCM technique.[22] Briefly, pretransplant sera from each recipient was incubated with freshly isolated lymphocytes from their respective donors and bound immunoglobulins were detected by the addition of a FITC-labelled anti-human pan-IgG F(ab')2 antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). T-cell and B-cell reactivity was determined by three-colour fluorescence using anti-CD3-PerCP and anti-CD19-PE antibodies (BD Biosciences, San Jose, CA, USA) on a BD FACSLyric™ system.

IgG subtype assay

Analysis of the IgG subtypes was carried out by flow cytometry. Briefly, PBMCs isolated from the donor blood samples were incubated with peptide Fc Receptor Blocker (Innovex Biosciences Inc., Richmond, CA, USA) for 10 min at room temperature followed by washing the cells with Dulbecco's phosphate-buffered saline (DPBS) (Lonza, Walkersville, MD, USA) and incubation with the patient sera for 30 min at 4°C. Donor cells were also incubated with negative control sera samples (Gemini Bio, West Sacramento, CA, USA). The cells were washed with wash buffer containing DPBS and 1% foetal bovine serum (FBS) (Gemini Bio™, West Sacramento, CA, USA). The washed cells were resuspended in wash buffer, added directly to the six-colour lyophilised antibody reagent, and incubated in the dark for 20 min. Cells were again washed with wash buffer and the specific anti-HLA IgG subtypes bound to the cells were detected using the multicolour flow cytometry detection method on BD FACSLyric™ system (BD Biosciences) and analysed using the BD FACSuite™ RUO v1.4 software.

There is a great degree of variation in the amount and type of antigens expressed on cells isolated from different donors leading to differences in the binding of each IgG subtype to the donor cells. To overcome this diversity, levels of each IgG subtype that bind donor cells incubated with negative human sera were used as base line. A threshold of twofold above these baseline levels for each IgG subtype was determined to be the cut-off value for positivity in patient sera exposed to the same donor cells.

To identify the specificity of each of the anti-human IgG subtypes to their primary target and demonstrate the absence of cross-reactivity among the antibodies themselves, individually coated IgG subtype control beads were incubated with the six-colour IgG subtype lyophilised mixture and analysed by flow cytometry.

Complement-dependent cytotoxicity crossmatch

The presence of complement-activating antibodies in the patient serum was detected using the CDC assay. Briefly, an immuno-magnetic separation technique was used to separately isolate T and B lymphocytes from whole blood, spleen or lymph nodes. The patient serum was heat inactivated and added to Terasaki crossmatch trays (One Lambda Inc.). The isolated T cells were added to the patient serum in the crossmatch trays and incubated for 30 min for T-cell CDC analysis. The cells were washed followed by the sequential addition of anti-human globulin (AHG) (One Lambda Inc.) and Class I complement (Cedarlane, Burlington, NC, USA) and incubated for 60 min at room temperature. The B-cell CDC analysis was performed by adding the isolated B cells to the patient serum in crossmatch trays. After an incubation of 30 min, the cells were washed followed by the addition of Class II complement (Cedarlane). The cells were then incubated for 45 min at room temperature. Acridine orange/ethidium bromide (AO/EB) quench reagent (One Lambda Inc.) was then added to both trays and observed under a fluorescent microscope. The presence of complement-activating antibodies in the patient serum was analysed by the amount of cell death caused by the activation of the complement pathway.

C3d assay

C3d analysis was carried out using the Luminex bead-based assay and the LIFECODES® C3d Detection assay (Immucor, Waukesha, WI, USA) as per the manufacturer's instructions. Briefly, recipient serum was incubated with bead-bound HLA antigens. A negative serum sample was added after this initial incubation as a source of complement. After washing the beads to remove unbound antibodies, they were incubated with anti-human C3d antibody conjugated to phycoerythrin. The beads were washed, diluted, and analysed in the Luminex® analyser. The signal intensity of the test sample was compared with the signal intensity of the beads exposed to the negative control sera to determine whether the test sample should be considered positive or negative for C3d bound to the antigen/antibody complex.

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AUTHOR CONTRIBUTIONS

Prakash N Rao: Conceptualization; funding acquisition; project administration; resources; supervision; writing – original draft; writing – review and editing. **Dayanand D Deo:** Conceptualization; data curation; formal analysis; investigation; methodology; project administration; resources; validation; visualization; writing – original draft; writing – review and editing. **Amitabh Gaur:** Methodology; resources; validation; writing – review and editing. **David A Baran:** Resources; writing – review and editing. **Mark J Zucker:** Resources; writing – review and editing. **Saurabh Kapoor:** Resources; writing – review and editing. **Misty A Marchioni:** Project administration; writing – review and editing. **Jesus Almendral:** Resources. **Praveen Kandula:** Resources. **Anup Patel:** Resources; writing – review and editing.

CONFLICTS OF INTEREST

The authors declare no 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 the article.