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Detection of complement-fixing and non-fixing antibodies specific for endothelial precursor cells and lymphocytes using flow cytometry

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Abstract

Donor human leukocyte antigen (HLA)-specific antibodies (Abs) with the ability to activate complement are associated with an increased risk of early Ab-mediated rejection (AMR) of kidney allografts. In recent years, also non-HLA Abs-binding endothelial cells have been shown to elicit early AMR. Donor-specific anti-endothelial cell Abs escape detection in the pre-transplant evaluation if only lymphocytes are used as target cells in crossmatch tests. We addressed whether endothelial precursor cells (EPCs) could be used for detection of complement-fixing as well as non-fixing Abs and if complement factor and immunoglobulin G (IgG) deposition on co-purified T and B cells correlated to the outcome of the T- and B-cell complement-dependent cytotoxicity assay. Deposition of complement factors C3c and C3d, but not C1q nor C4d, were detected on EPCs and lymphocytes upon incubation with HLA Ab-positive sera. There was a correlation between the amount of C3c deposition and IgG binding on EPCs ($R^2 = 0.71$, $P = 0.0012$) and T cells ($R^2 = 0.74$, $P = 0.0006$) but not for B cells ($R^2 = 0.34$, $P = 0.059$). The specificity and sensitivity for C3d deposition on endothelial precursor cell crossmatch (EPCXM) T cells *vs* the T complement-dependent cytotoxicity (CDC) assay were 69% and 72%, respectively. The EPCXM B-cell C3d assay had considerably lower sensitivity (39%) than the B CDC assay. Altogether, this novel assay based on the detection of complements factors on EPCs and lymphocytes by flow cytometry may widen the diagnostic repertoire and thereby improve the clinical management of patients undergoing kidney transplantation.

Introduction

Preformed complement-fixing donor human leukocyte antigen (HLA)-specific antibodies (Abs) (HLA DSA) induce kidney allograft rejections (1). Therefore it is of outmost importance to perform pre-transplant testing for the presence of potentially harmful HLA Abs (2). The method of choice for the last 40 years is the complement-dependent cytotoxicity (CDC) assay (3, 4). The CDC has become the 'golden standard' in crossmatch testing and has clearly served its purpose. However, the technique has limited sensitivity, a subjective read-out and a high frequency of false-positive tests due to non-HLA Abs, auto-Abs and/or non-deleterious IgM Abs (reviewed in (4)). Several modifications of the assay have been introduced over the years in order to avoid some of these problems (5–8). In addition the relevance of using cytolysis as read-out for complement activation has been questioned (9). It has recently been shown that detection of complement factors deposited on lymphocytes could be a more accurate way of determining HLA Ab-triggered complement activation (10, 11). One of the major drawbacks of the CDC assay, as it is designed today, is that it only detects Ab reactivity to antigens expressed on T and B lymphocytes. It is known from several studies that also non-HLA Abs (e.g. anti-endothelial cell Ab; AECA), not detectable in regular lymphocyte crossmatch tests, play a role in graft rejection (12–16). Three cases of kidney graft rejection caused by non-HLA-specific Abs at our Tx center led to the development of a novel cell-based crossmatch assay for the determination of donor-specific AECA (17–19). Since 2007, we routinely use this endothelial precursor cell crossmatch (EPCXM) assay in the selection of living kidney donors ((20), M.Alheim, manuscript in preparation). Recently, we showed that the EPCXM can be

used for simultaneous detection of Abs against donor EPCs and lymphocytes (21). Along this path of method development we here addressed whether the EPCXM could be used for the detection of complement-fixing Abs. A multicolor flow cytometric crossmatch assay was evaluated using EPCs and lymphocytes isolated on magnetic nanoparticles carrying anti-Tie-2 Abs as target cells. Deposition of complement factors was used as the determinant of Ab-induced complement activation.

Materials and methods

Cells and human sera

Cells were isolated from peripheral blood of healthy individuals. HLA Ab-negative and -positive sera were from patients on the wait list for kidney transplantation. The HLA Ab immunoglobulin G (IgG) reactivity of the sera was determined by FlowPRA®, LABScreen® PRA or single antigen assay (One Lambda Inc., Canoga Park, CA). A panel of 42 different sera were grouped into four separate categories based on their panel-reactive Ab (PRA) reactivity; HLA I−/II− (0% PRA; $n = 13$), HLA I−/II+ (9–77% PRA; $n = 6$), HLA I+/II− (13–87% PRA; $n = 4$), HLA I+/II+ (6–100% PRA; $n = 19$). AB serum from male blood donors were used as negative control serum (NS). Pooled serum from highly immunized patients >70% PRA was used as positive control serum (PS). Human sera used as source of complement were from healthy individuals. The presence of HLA Abs of IgM class was determined by the LABScreen® Mixed assay using phycoerythrin (PE)-conjugated secondary donkey anti-human IgM Ab (Jackson ImmunoResearch Europe Ltd, Suffolk, UK).

Isolation of EPCs and peripheral blood mononuclear cells

Tie-2^+ EPCs were isolated with the commercially available kit, XM-ONE® (AbSorber AB, Stockholm, Sweden), as previously described (20). T and B cells co-purified with EPCs are hereafter referred to as EPCXM T cells and EPCXM B cells, respectively (21). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Lymphoprep™; Axis-Shield PoC AS, Oslo, Norway).

Measurement of complement deposition by flow cytometry

PBMCs and EPCs were incubated with 50 µl of HLA Ab-NS, HLA Ab-PS or patient sera (HLA Ab − or +) for 30 min in room temperature. Cells were washed twice with PBS + 0.1% bovine serum albumin (BSA). Fifty microliters of complement-active normal human serum or heat-inactivated serum (56°C, 30 min) with reduced complement activity were added as source of complement to target cells and incubated at 37°C for 20 min. Thereafter the cells were washed three times with PBS + 0.1% BSA and stained with one of the following FITC-conjugated complement-specific Abs; C1q (F0254; Dako, Glostrup, Denmark), C3c (F0201; Dako), C3d (F0323; Dako), C4d (12–500; American Research Products, Waltham, MA) or Alexa Fluor® 488-conjugated C3d (A207; Quidel Corporation, San Diego, CA). The latter Ab was conjugated with Alexa Fluor® 488 using a commercial labeling kit (A-30006; In Vitrogen Corporation, Carlsbad, CA). The labeling procedure was done according to instructions provided by the manufacturer. To measure IgG Ab-binding, secondary PE-goat anti-human IgG (109-116-098; Jackson ImmunoResearch Europe Ltd) was added. For identification of T and B cells, samples were stained with CD3-PC5 (A07749; Beckman Coulter, Miami, FL) and CD19-PC7 (IM3628; Beckman Coulter). Fifty microliters of the Ab staining mixture (anti-complement factor Abs, secondary anti-IgG, anti-CD3 and anti-CD19) were added to cells and incubated for 20 min on ice and in the dark. Cells were then washed once and resuspended in 300 µl PBS + 0.1% BSA and immediately analyzed on a Beckman Coulter FC500 instrument using the CXP software. During analysis, gates were set on T cells (SSC/CD3+), B cells (SSC/CD19+) and EPC [forward scatter(FSC)/side scatter(SSC)]. The complement factor deposition and IgG binding were expressed as the fluorescence ratio for test serum/negative serum using linear values. The cut-off for IgG and complement factors were determined separately for C3c and C3d using multiple sera from non-immunized male individuals against cells from several donors. Negative/positive cut-off for C3c/IgG experiments were as follows: EPCs 1.7/2.2; EPCXM T cells 2.3/1.7; EPCXM B cells 1.7/2.0. Negative/positive cut-off for C3d/IgG experiments were as follows: EPCs 1.3/1.4; EPCXM T cells 1.3/1.3; EPCXM B cells 1.6/2.9. In indicated experiments, the frequencies of cells with disrupted cell membrane integrity, as a sign of necrosis, were determined by addition of propidium iodide (P4170; Sigma-Aldrich Corporation, St. Louis, MO).

Complement-dependent cytotoxicity

The standard CDC assay was performed as described (2). T and B lymphocytes were isolated from whole blood of donors by anti-CD8 and anti-CD19-conjugated magnetic beads (Life Technologies Corporation, Carlsbad, CA), and were used as target cells in the CDC. Rabbit complement was used at a pre-determined optimal dilution for cell lysis (Cedarlane Laboratories Ltd, Ontario, Canada). Lysis of >20% of the cells compared to the background lysis with an NS was considered as positive.

Statistical analyses

The two-tailed Fischer's exact test or Student's *t*-test was used for statistical analysis. A *P*-value of <0.05 was considered significant. Statistical calculations were performed with the MICROSOFT EXCEL ANALYSE-IT® software.

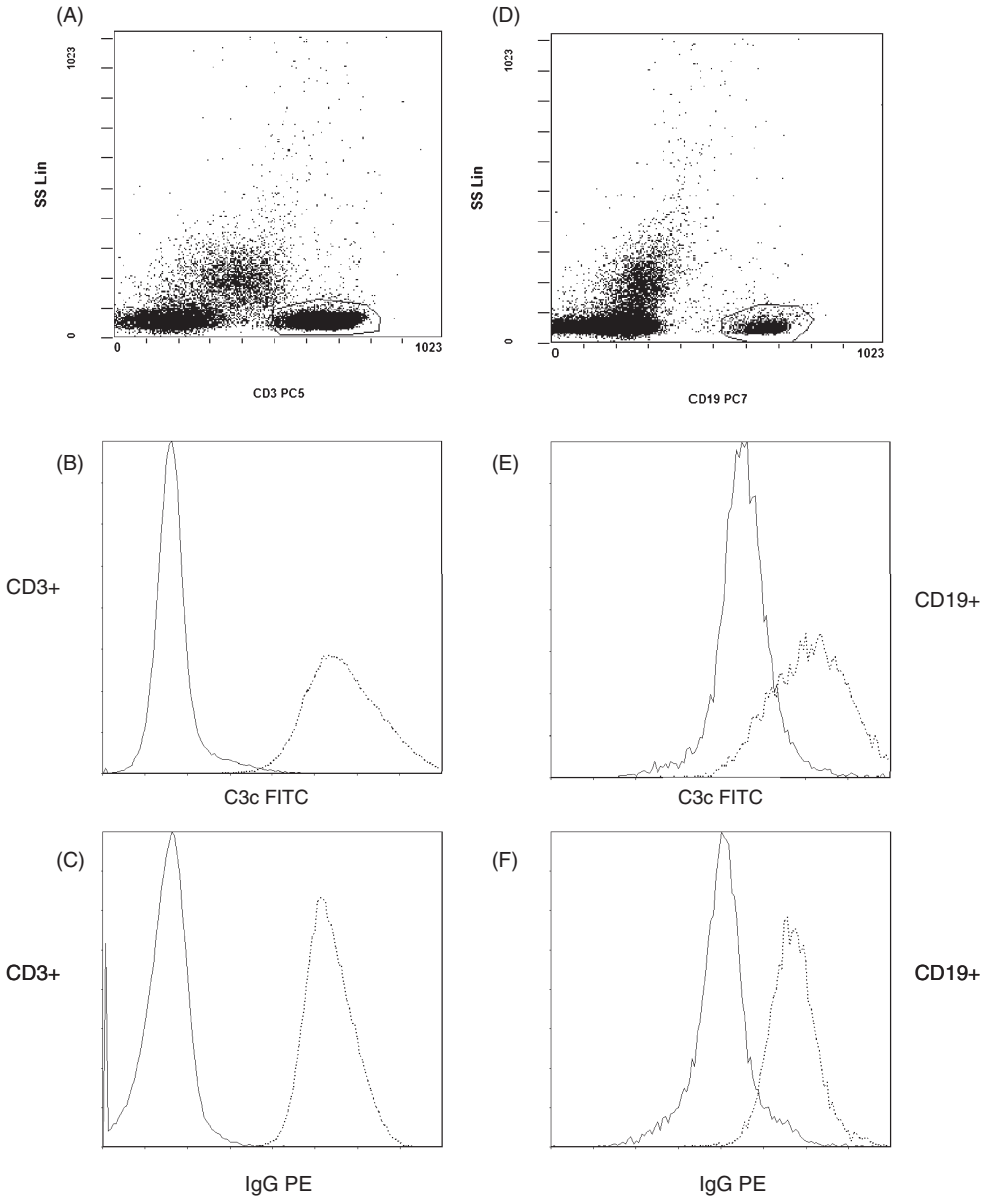

Figure 1 Flow cytometric detection of complement factor C3c and immunoglobulin G (IgG) deposition on peripheral blood mononuclear cells (PBMC) gated on CD3+ T cells (A–C) and CD19+ B cells (D–F). PBMC were incubated with human leukocyte antigen (HLA) antibody-negative control serum (NS; solid line) and HLA antibody-positive control serum (PS; dotted line).

Results

Complement factor deposition on EPCs

With a previously described flow cytometric assay we initially verified that human complement factors are deposited on the surface of peripheral blood T and B cells upon binding of complement-fixing HLA Abs (Figure 1; (10, 11)). Several different Abs specific for human complement were tested (see *Materials and methods*). Generally the C3c and C3d Abs resulted in the strongest fluorescence signal. The Abs directed against C1q and C4d resulted in a modest signal with fluorescence ratios barely above 1 (data not shown). Apart from detection of complement factors, the assay supports simultaneous detection of IgG as depicted in Figure 1C, F. Next, we set-out to determine whether this type of complement-binding assay also could be applied on EPC isolated with the XM-ONE® kit. A set of pilot experiments ($n = 10$) with Tie-2^+ cells isolated from peripheral blood showed that complement factors C3c and C3d are deposited on EPCs (Figure 2A, B) and co-purified lymphocytes (Figure 2A, C). C1q and C4d (data not shown) were not detected at any substantial levels. Use of the viable/dead cell

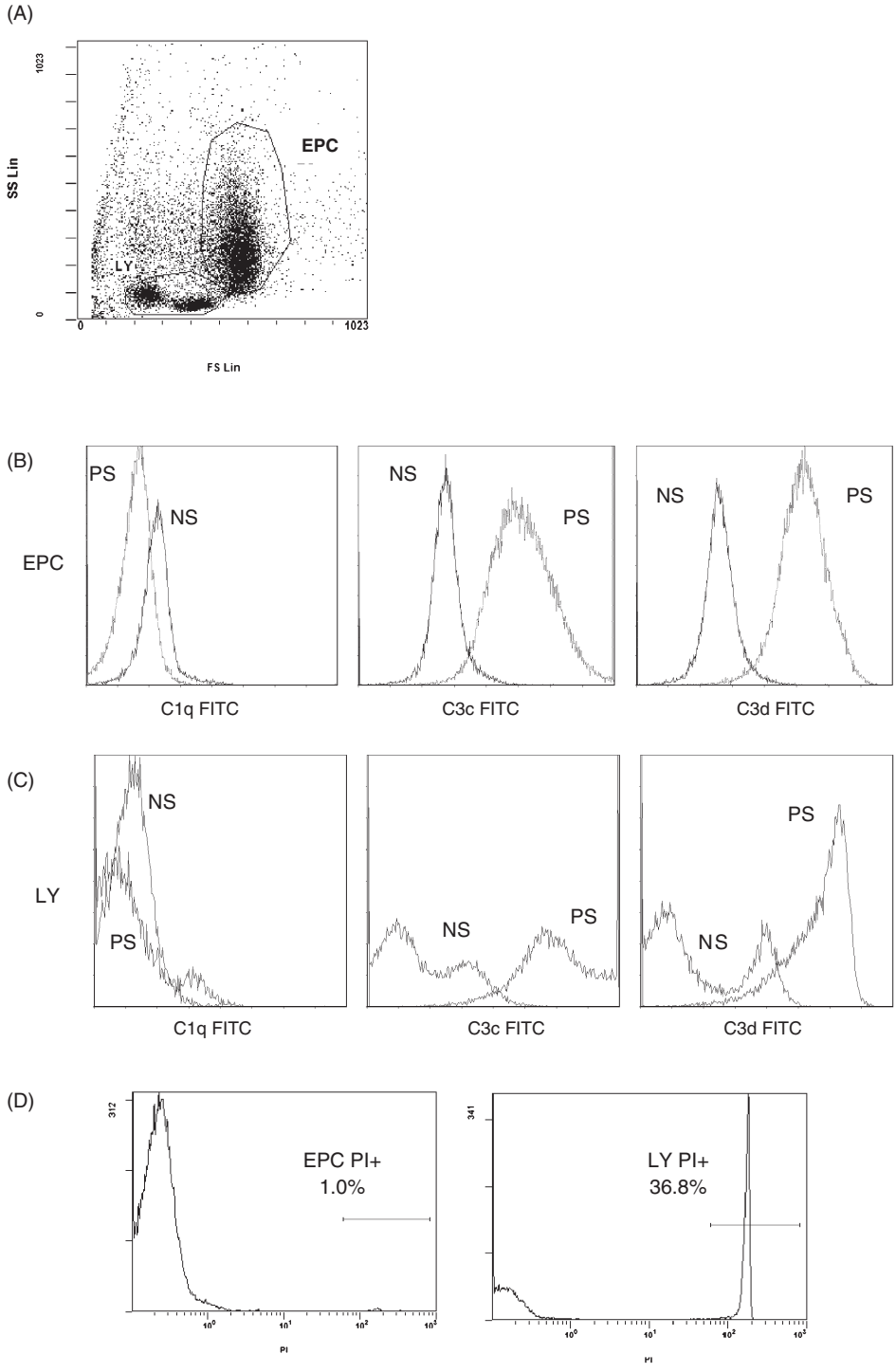

Figure 2 Flow cytometric detection of complement factors on endothelial precursor cells with low induction of cytolysis. Endothelial precursor cells (EPCs) and co-purified lymphocytes (LY) isolated with anti-Tie-2 antibody (Ab)-conjugated beads were incubated with human leukocyte antigen (HLA) Ab-negative control serum (NS) and HLA Ab-positive control serum (PS) and stained with C1q, C3c and C3d Abs. The EPCs (B) and lymphocytes (C) were FSC/SSC gated and the deposition of the complement factors C1q, C3c and C3d was determined. The frequencies of non-viable [propidium iodide (PI)+] EPCs and lymphocytes after incubation with HLA Ab-positive control serum are shown in (D). One representative experiment is shown.

exclusion dye, propidium iodide (PI), showed that human serum or rabbit serum used as source of complement induced only modest necrosis of endothelial cells (Figure 2D and data not shown). In contrast, for the lymphocytes (co-purified with EPC) cytolysis were induced resulting in 30%–50% dead cells upon exposure to serum with complement-fixing HLA Abs (Figure 2D). Higher frequencies of dead lymphocytes (50%–80%) were observed when rabbit serum was used as complement source (data not shown).

Heat inactivation of complement reduce deposition of complement factors

To verify that the deposition of complement factors on the surface of cells was dependent on active complement, the assay was performed with heat-inactivated serum (56°C, 30 min); a procedure known to abolish endogenous complement activity (22). The levels of C3c on the surface of EPC and the percentage of necrotic cells (PI+) were reduced after heat-inactivation (HI) of human serum (Figure 3A). Similar results were observed for co-purified lymphocytes (Figure 3B). The levels of C3d were also reduced on EPC and co-purified lymphocytes after serum heat inactivation (Figure 3C).

Simultaneous detection of complement factors and IgG on cells immunomagnetically isolated on beads carrying anti-Tie-2 Abs

As described previously (21) and confirmed in this study, T and B lymphocytes are co-purified upon isolation of Tie-2$^+$ EPC using the XM-ONE® kit. A large number of experiments ($n = 39$) were performed in order to establish whether the cells isolated on Tie-2 Ab-bearing magnetic beads could be used as targets in a flow cytometric method to assess the deposition of complement factors as well as IgG binding. Target cells were isolated from different donors and incubated with HLA Ab− and HLA Ab+ serum. The mean values for complement factor deposition and IgG binding are shown in Table 1. The fluorescence ratio for the complement factor C3d using undiluted HLA Ab+ serum were for EPC and B cells generally around 2–4. Considerably higher fluorescence ratios of complement factors were found on T cells. The level of C1q and C4d deposition was consistently low on all target cells. In addition, the levels of IgG binding were highest on T cells followed by B cells and EPC. Furthermore, there was a correlation between the amount of C3c deposition and IgG binding on EPCs (Figure 4A; $R^2 = 0.71$; $P = 0.0012$) and T cells (Figure 4B; $R^2 = 0.74$; $P = 0.0006$). The coefficient of determination was considerably lower on B cells ($R^2 = 0.34$) and did not reach statistical significance ($P = 0.059$) (Figure 4C). Serial dilution of HLA Ab+ serum resulted in reduced C3c (Figure 5A) and C3d (Figure 5B) deposition. The level of complement deposition on T cells was significantly reduced already at a twofold serum dilution. A minor reduction of C3c deposition

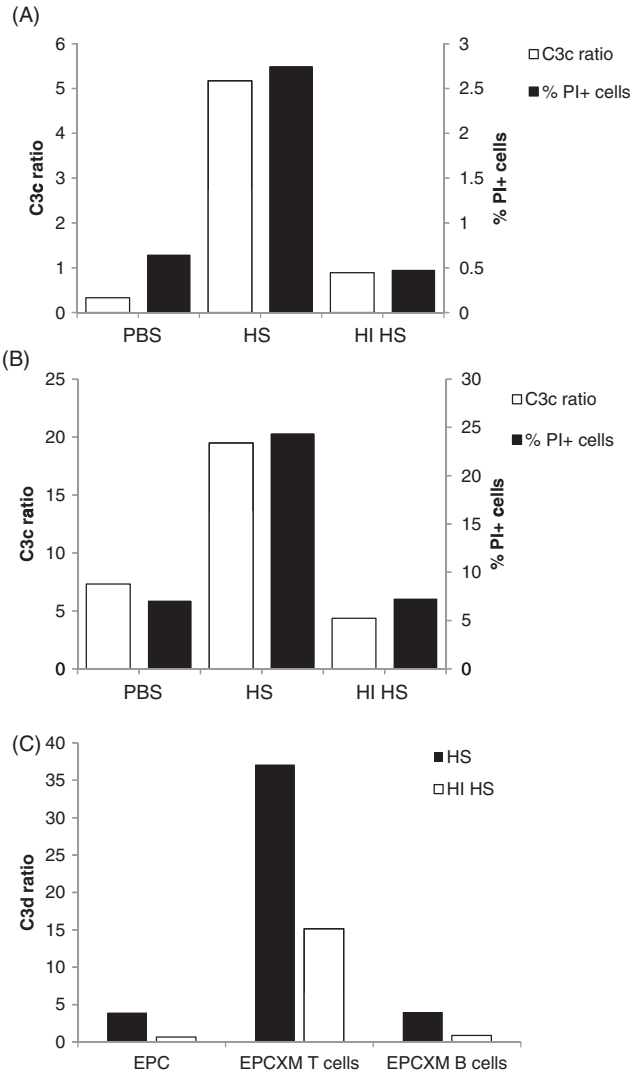

Figure 3 Heat inactivation of complement reduces deposition of complement factors and cytolysis. Endothelial precursor cells (EPCs) (A) and co-purified lymphocytes (B) isolated with anti-Tie-2 antibody-conjugated beads were incubated with phosphate-buffered saline (PBS), human serum (HS) or heat-inactivated human serum (HI HS) as the source of complement. The C3c ratio (MFI-positive/negative serum; white bar) and % propidium iodide (PI)+ cells (black bar) were determined. C3d ratio for EPC, endothelial precursor cell crossmatch (EPCXM) T cells and EPCXM B cells with HS and HI HS (C).

was observed on EPC and B cells at this dilution. The reduction of IgG binding on EPC, T and B cells were not as pronounced as for C3c and C3d (Figure 5). Even at a 1:8 dilution, high levels of IgG were detected on the cell surface.

Comparison of complement factor deposition on EPCs and the T- and B-cell CDC assay

The EPC complement deposition and IgG assay and the conventional CDC assay were compared side-by-side in

Table 1 Simultaneous detection of complement factors and IgG on EPCs, EPCXM T and EPCXM B cells[a]

	C3c	C3d	C1q	C4d	IgG
EPC	2.1 ± 0.5	3.4 ± 1.4	0.6 ± 0.4	0.7 ± 0.5	5.0 ± 2.2
EPCXM T cells	43.9 ± 25.1	46.7 ± 23.3	0.9 ± 0.4	1.6 ± 0.9	28.3 ± 20
EPCXM B cells	1.7 ± 0.5	4.2 ± 1.3	1.4 ± 0.8	1.8 ± 0.6	8.7 ± 3.9

EPC, endothelial precursor cell; EPCXM, endothelial precursor cell crossmatch; IgG, immunoglobulin G.

[a]Results are expressed as a fluorescence ratio (mean ± SD) of HLA Abs-positive serum over negative serum. C3c ($n = 11$), C3d ($n = 4$), C1q ($n = 3$) and C4d ($n = 3$). IgG represents mean ratio ± SD from all experiments ($n = 21$). Target cells were from different donors.

10 separate experiments. Target cells from different donors ($n = 9$) were crossmatch tested against HLA class I and II Ab-negative and -positive sera from patients on the kidney transplant waiting list. In total 47 different target cell/serum combinations were tested (Table 2). The outcome of the EPCXM following detection of C3d/IgG deposition was compared with the outcome of the CDC assay (Figure 6). The CDC crossmatch outcome was grouped as: CDC T−/B− ($n = 20$), CDC T−/B+ ($n = 9$), CDC T+/B− ($n = 1$), CDC T+/B+ ($n = 17$). The majority (14/20, 70%) of the CDC T−/B− crossmatch tests were EPC C3d−. Thirty per cent (6/20) of the target/serum combinations that were CDC T−/B− resulted in C3d deposition on EPC. Four of the 6 (67%) sera resulted in IgG binding. However, none of these sera were found to have HLA class I or II Abs as determined by solid-phase Ab screening (data not shown). Furthermore, tests with CDC T−/B+, CDC T+/B− and CDC T+/B+ sera resulted in 44%, 100% and 59% EPCs with C3d deposition, respectively. In the latter T+/B+ CDC group, 88% (15/17) of the target/serum combinations resulted in IgG binding on EPCs.

Comparison of complement factor deposition on T and B cells co-purified with EPCs and the T- and B-cell CDC assay

EPCXM T and B cells co-purified with EPCs can be used for simultaneous detection of C3d and IgG on all three cell subsets. Therefore, we addressed whether there was any correlation between the T and B cell CDC assay and the C3d assay when gating on EPCXM T and B cells. Seventy-two per cent (13/18) of the T CDC+ tests were T cell C3d+. Ten of the 13 (77%) were IgG+. Notably, all three T C3d+/IgG− within the T CDC− group had a C3d ratio just above cut-off (data not shown). Twenty-nine of 47 target cell/serum combinations resulted in a negative T cell CDC (T CDC−; Table 3). Sixty-nine per cent (20/29) of these were negative with regard to C3d deposition on T cells co-purified with EPCs. Of the C3d+ T-cell crossmatch tests that were negative in the T CDC, 67% were IgG positive. The specificity and sensitivity for the

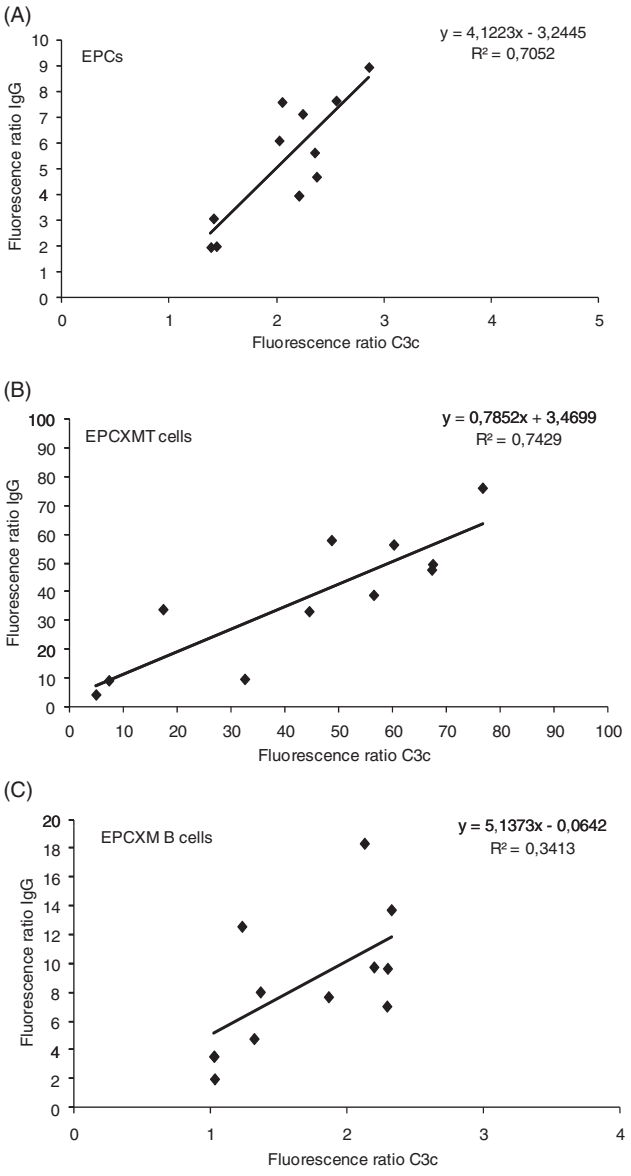

Figure 4 Relationship between the amount of complement factor deposition (C3c) and immunoglobulin G (IgG) binding on endothelial precursor cells (EPCs), endothelial precursor cell crossmatch (EPCXM) T cells and EPCXM B cells. Linear regression analysis of C3c deposition *vs* IgG binding on EPCs (A; $n = 11$), EPCXM T cells (B; $n = 11$) and EPCXM B cells (C; $n = 11$). Each data point represents a separate experiment with a given donor/serum combination. Different donors were used in each experiment. The same negative and positive control serum was used throughout the experiments.

flow cytometric assessment of C3d deposition on EPCXM T cells *vs* the T CDC assay were 69% and 72%, respectively.

Eighty-six per cent (18/21) of the B CDC− were C3d− on EPCXM B cells. Thirty-eight per cent (10/26) of the B CDC+ were C3d+ on EPCXM B cells. Nine of the 10 (90%) were also IgG+. In tests that were B CDC+/EPCXM B cell C3d− the percentage of cell lysis in the B CDC ranged

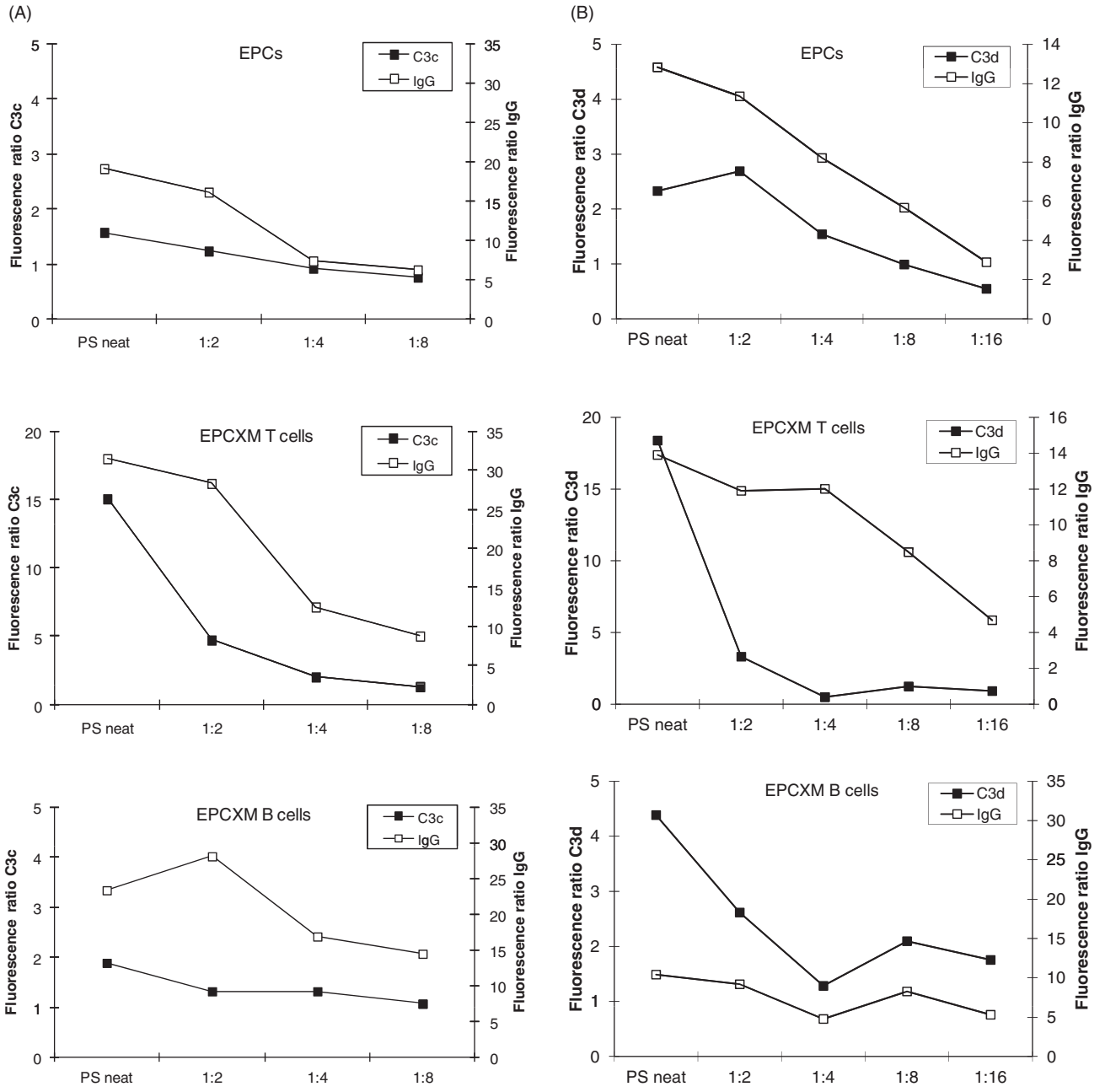

Figure 5 Relationship between deposition of C3c (A), C3d (B) and immunoglobulin G (IgG) binding on endothelial precursor cells (EPCs), endothelial precursor cell crossmatch (EPCXM) T and EPCXM B cells at different serum dilutions. Results are expressed as a fluorescence ratio of human leukocyte antigen antibodies-positive serum over negative serum. Data are from representative experiments.

between 25% and 100%. In the majority (11/16; 69%) of tests, at least 50% lysis was observed (data not shown). Notably, in 5 of those 11 tests (45%) no IgG deposition on B cells was observed. The majority of sera (6/8) that were B CDC+ and EPCXM B cell C3d−/IgG− were also negative for HLA Abs of IgM class (data not shown). The flow cytometric EPCXM B cell C3d assay had high specificity (86%), but considerably lower sensitivity (39%) than the B CDC assay.

The width of the panel-reactive Ab repertoire and its impact on C3d/IgG deposition and CDC

The panel of sera ($n = 42$) were grouped into four separate categories based on the PRA reactivity; HLA I−/II− (0% PRA), HLA I−/II+ (9%–77% PRA), HLA I+/II− (13%–87% PRA), HLA I+/II+ (6%–100% PRA). The HLA I−/II− group of sera resulted in 31% EPC C3d+, 13% EPCXM T cell C3d+ and 13% EPCXM B cell C3d+ crossmatch tests

Table 2 Complement factor deposition and IgG binding on T and B cells co-purified with endothelial precursor cells and the T- and B-cell complement-dependent cytotoxicity assay[a]

		EPCXM T cells			EPCXM B cells			EPC	
Crossmatch no.	Serum PRA class I/II (%)	C3d	IgG	T CDC (% lysis)	C3d	IgG	B CDC (% lysis)	C3d	IgG
1	0/0	2.0	3.1	10	1.3	2.0	5	1.8	5.7
2	0/0	1.7	2.9	10	1.2	1.9	5	1.5	4.6
3	0/0	0.9	1.3	10	0.4	0.8	10	0.6	1.1
4	0/0	0.9	1.2	10	0.3	0.7	10	0.8	0.6
5	0/0	1.1	1.0	5	0.9	0.9	15	0.8	1.1
6	0/0	1.2	1.0	15	1.4	1.2	15	1.3	1.1
7	0/0	0.9	1.7	10	2.5	7.2	20	3.9	7.8
8	0/0	1.0	0.9	10	1.1	0.3	20	0.8	1.4
9	0/0	0.5	1.6	20	2.0	8.8	20	1.7	8.0
10	0/0	1.2	0.9	20	1.3	1.4	20	0.9	1.0
11	0/0	1.1	1.0	5	0.9	1.2	25	0.8	1.1
12	0/0	0.9	0.8	5	1.0	0.6	40	0.9	0.7
13	0/0	1.0	1.0	10	1.0	1.3	80	1.3	1.1
14	0/0	0.9	0.8	10	1.1	0.8	10	1.0	1.3
15	0/0	0.9	0.9	15	1.1	1.2	5	1.0	0.8
16	0/0	0.8	0.7	10	0.8	0.4	10	0.8	0.9
17	0/57	1.4	2.2	10	0.9	5.4	10	0.9	1.5
18	0/43	1.1	0.9	5	1.0	1.4	20	1.0	1.1
19	0/71	2.2	4.5	5	1.3	2.7	25	1.3	1.2
20	0/40	0.4	1.7	20	1.8	9.4	30	1.8	8.1
21	0/40	0.9	0.8	20	0.7	0.7	75	0.8	0.9
22	0/77	1.2	1.1	60	1.2	1.7	60	0.9	1.8
23	0/77	0.5	1.7	90	2.0	17.9	80	2.0	8.4
24	0/9	0.9	0.8	10	1.3	0.7	15	0.9	1.2
25	49/0	1.0	0.8	10	1.1	1.3	35	2.1	0.9
26	13/0	1.0	0.9	20	0.7	0.7	10	1.0	1.1
27	16/0	0.7	0.8	5	0.6	0.3	10	0.9	0.9
28	87/0	0.9	6.2	25	1.1	2.1	50	0.8	1.8
29	73/83	1.2	1.2	15	0.5	0.9	15	1.7	1.2
30	75/83	1.4	3.2	15	1.2	2.5	20	0.8	0.7
31	67/89	1.1	7.8	40	0.9	6.4	30	1.1	2.3
32	65/66	0.5	4.8	30	2.0	17.2	50	4.0	8.4
33	99/76	1.1	10.1	50	0.8	2.1	60	1.0	1.0
34	84/94	1.8	0.9	60	0.9	1.4	90	0.9	1.0
35	98/94	1.9	28.8	40	1.0	20.6	100	0.9	3.0
36	85/100	54.4	35.2	80	3.4	9.7	100	4.0	5.5
37	98/94	96.6	12.0	90	10.8	5.9	100	11.7	5.1
38	65/63	1.8	7.7	90	0.6	2.9	100	0.5	1.5
39	93/97	40.6	36.5	100	12.8	40.6	100	19.4	78.9
40	96/100	34.1	11.4	100	3.5	13.3	100	2.6	6.3
41	89/83	1.8	25.6	70	0.8	20.0	70	0.9	8.9
42	75/89	18.6	0.8	100	2.8	0.5	100	1.7	4.5
43	6/61	1.0	4.1	10	1.1	4.4	60	1.1	1.5
44	85/100	3.2	2.9	80	7.4	8.5	100	8.4	35.5
45	67/86	1.7	6.4	50	1.1	9.0	20	1.9	7.3
46	84/26	23.0	26.4	100	1.0	15.5	100	1.1	3.9
47	84/86	1.2	4.3	20	1.5	3.1	30	1.7	4.5

CDC, complement-dependent cytotoxicity; EPC, endothelial precursor cell; EPCXM, endothelial precursor cell crossmatch; IgG, immunoglobulin G; PRA, panel-reactive Ab.

[a]Data from all crossmatches ($n=47$). Serum PRA class I and II (%) for each crossmatch are shown together with C3d deposition and IgG binding. CDC lysis >20% were considered as positive.

(Table 4). Nineteen per cent (3/16) of the tests were B-cell CDC+. None (0/16; 0%) of the tests were CDC T+. The HLA I−/II+ group of sera resulted in similar frequencies of EPC C3d+, EPCXM T cell C3d+ and EPCXM B cell C3d+ crossmatch tests. A high frequency of tests (5/8; 63%) was B CDC+ in the HLA I−/II+ group of sera. Furthermore, as expected, the highest number of positive crossmatch tests was observed with HLA class I and II positive sera. Apart from the EPCXM B cell C3d assay with only 37% positive crossmatch tests, the frequencies of C3d+, IgG+ and

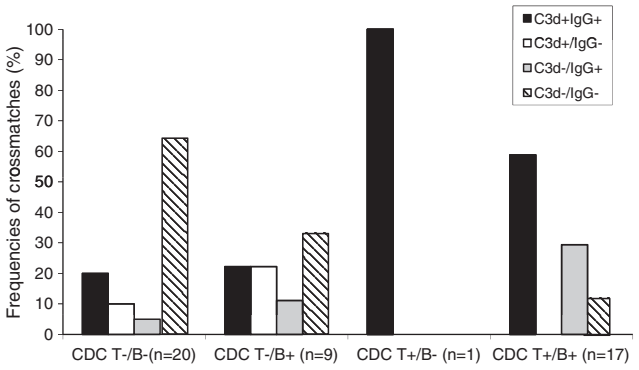

Figure 6 The frequency of complement factor and immunoglobulin G (IgG) deposition on endothelial precursor cells and its relationship to the outcome of the T- and B-cell complement-dependent cytotoxicity assay. The frequency of C3d and IgG-positive and -negative endothelial precursor cells (EPCs) within each complement-dependent cytotoxicity (CDC) group is shown.

Table 4 The frequency of C3d and IgG deposition, T- and B-cell cytotoxicity and their dependence on panel-reactive antibody reactivity

C3d+[a]	HLA I−/II−	HLA I−/II+	HLA I+/II−	HLA I+/II+
EPC	5/16 (31%)	3/8 (38%)	1/4 (25%)	10/19 (53%)
EPCXM T cells	2/16 (13%)	2/8 (25%)	0/4 (0%)	13/19 (68%)
EPCXM B cells	2/16 (13%)	2/8 (25%)	0/4 (0%)	7/19 (37%)
IgG+				
EPC	4/16 (25%)	4/8 (50%)	1/4 (25%)	15/19 (79%)
EPCXM T cells	4/16 (25%)	4/8 (50%)	1/4 (25%)	16/19 (84%)
EPCXM B cells	2/16 (13%)	2/8 (25%)	0/4 (0%)	13/19 (68%)
CDC+				
T cells	0/16 (0%)	2/8 (25%)	1/4 (25%)	15/19 (79%)
B cells	3/16 (19%)	5/8 (63%)	2/4 (50%)	16/19 (84%)

CDC, complement-dependent cytotoxicity; EPC, endothelial precursor cell; EPCXM, endothelial precursor cell crossmatch; IgG, immunoglobulin G; PRA, panel-reactive Ab.
[a]Number of C3d+, IgG+ and CDC+ crossmatches within each PRA group. The percentage is shown in parenthesis.

CDC+ were all above 50% for the HLA class I and II positive sera. Interestingly, a high frequency of HLA I+/II+ sera (13/19; 68%) generated IgG+ EPCXM B cells but only 37% of the sera induced C3d deposition. In this group of sera there was significantly fewer EPCXM B cell C3d+ crossmatch tests than B cell CDC+ tests (7/19 *vs* 16/19; $P = 0.007$).

Discussion

In this study, we addressed whether the XM-ONE® kit can be used to determine Ab-induced complement factor deposition on EPCs as well as on co-purified T and B cells by multicolor flow cytometry. The concept of measuring deposition of complement components and anti-HLA class I and II IgG Ab binding on PBMCs have previously been described by Scornik and co-workers (10, 11). Initially, we performed pilot tests with cells isolated on Tie-2 Ab-bearing magnetic beads in order to optimize the conditions and to ensure the validity of the assay. Several different HLA Ab-negative and -positive sera as well as different sera serving as a complement source were tested (data not shown). Negative and positive cut-off values for complement factor deposition and IgG binding were also determined. Furthermore, both polyclonal (rabbit anti-human) and monoclonal (mouse anti-human) Abs specific for different human complement factors were tested. We found that deposition of C3d was the best biomarker on the cell surface of EPCs, T and B cells for complement activation. The C3c marker generally resulted in low deposition in particular for EPC and B cells. C1q and C4d were deposited to a much lower degree for all target cells. The finding that C1q deposition is hard to detect is in line with previous reports in which PBMCs served as target cells (11). One explanation is that the experimental conditions do not facilitate detection of early markers such as C1q (or C4d). Further, because of the amplification of complement factor cleavage mediated by the C3 convertase (C4b-C2a), the concentration on the cell surface of complement fragments derived from C3 is likely to be higher than that of factors earlier in the cascade. However, it is important to note that we did not test additional clones of C1q or C4d Abs nor did we include an internal positive control (apart from HLA Ab-positive serum) to verify that these two Abs were suitable for flow cytometry as stated by the vendors. The results for C1q and C4d should therefore be interpreted with caution.

Table 3 The frequency of C3d and IgG deposition on EPCXM T and B cells, and its dependence on T- and B-cell complement-dependent cytotoxicity outcome

	EPCXM T cells[a]				CDC *vs* EPCXM C3d	
	C3d+/IgG+	C3d+/IgG−	C3d−/IgG+	C3d−/IgG−	Specificity (95% CI)	Sensitivity (95% CI)
T CDC− ($n = 29$)	6/29 (21%)	3/29 (10%)	4/29 (14%)	16/29 (55%)	0.69 (0.49–0.85)	0.72 (0.47–0.90)
T CDC+ ($n = 18$)	10/18 (55%)	3/18 (17%)	5/18 (28%)	0/18 (0%)	—	—
	EPCXM B cells					
B CDC− ($n = 21$)	2/21 (10%)	1/21 (5%)	3/21 (14%)	15/21 (71%)	0.86 (0.64–0.97)	0.39 (0.20–0.59)
B CDC+ ($n = 26$)	9/26 (35%)	1/26 (4%)	8/26 (31%)	8/26 (31%)	—	—

CDC, complement-dependent cytotoxicity; EPC, endothelial precursor cell; EPCXM, endothelial precursor cell crossmatch; IgG, immunoglobulin G.
[a]Frequencies of C3d/IgG-negative and -positive crossmatches within each CDC-negative and -positive group.

In the early phase of this study during the optimizing of the assay conditions, purified rabbit complement was tested as complement source. Interestingly, no lysis of EPCs was detected in any of the experiments irrespective of the length of incubation time (up to 1 h; data not shown). In contrast, a large fraction (50%–80%) of the co-purified lymphocytes was lysed. Furthermore, only very weak lysis of EPCs was observed with human serum as complement source. These data suggest that the frequency of necrotic cells as read-out will not be suitable to asses Ab-induced complement activation on isolated EPCs. This observation is in line with the notion that endothelial cells lining the graft are relatively more resistant to Ab-induced complement-mediated lysis than other cells of the graft; cells not normally exposed to blood (23). Endothelial cells are known to express a plethora of complement-regulatory proteins (CRP) such as CD59, CD46 and CD55, which can protect against complement-induced lysis (24–26). The expression level of complement-inhibitory molecules on EPCs is yet to be determined. However, we hypothesize that CRPs are expressed on Tie-2^+ EPCs and that they may influence complement factor deposition and susceptibility to lysis. The level of C3c/d deposition on EPCs and EPCXM B cells was in general significantly lower than on EPCXM T cells. Whether the relatively low C3c/d ratio on EPCs and EPCXM B cells is due to higher background staining generating a lower signal-to-noise ratio compared to T cells and/or due to other factors such as the repertoire or density of CRPs expressed is presently unknown. It is well-known that B cells have an intrinsically higher background staining in part because of Fc-receptor expression (27). Fc-receptors are expressed on Tie-2^+ EPCs (Maria Sundbäck, personal communication, 18 January 2012).

We found a correlation between the level of IgG and C3c deposition with a given negative/positive serum combination against target cells isolated from different donors. The positive serum predominantly contained IgG Abs specific for HLA class I and II. Only low or modest levels of HLA IgM Abs were detected (data not shown). Interestingly, C3c deposition sharply dropped upon serum dilution. In contrast, the IgG binding was not affected to the same extent and was still above cut-off after several dilutions. These findings are in line with previous data by Scornik et al. (11). An appealing idea, presented by this group, is to use C3 deposition as a tool to monitor the efficacy of patient desensitization (e.g. following immunoadsorption and plasma exchange). Our experience is that determination of IgG levels by solid-phase techniques after pre-treatment strategies rarely give any conclusive results. In most cases IgG is readily detected even after several rounds of Ab removal.

In our side-by-side comparison of the EPCXM C3d deposition assay and the CDC assays, we found that a considerable number (11/27; 41%) of T and/or B CDC+ tests did not show up as positive for C3d. In particular, the comparison between B CDC and the C3d deposition on B cells co-purified with the EPCs showed a low degree of concordance (see Table 3). This could either be explained by a high proportion of false-positive CDC tests or a poor sensitivity of the flow cytometric C3d assay. As shown in Table 4, the HLA I+/II− group did not result in any EPCXM T or B cell C3d+ crossmatch. Only one serum supported IgG deposition on EPCs and T cells. The HLA class I specificity determination of those four sera showed that three of them had rather low levels of HLA IgG Abs with mean fluorescence intensity (MFI) values below 3000 in the LabScreen® single antigen assay. Two of the sera resulted in positive CDC B-cell crossmatch tests. However, none of them induced IgG deposition on EPCXM B cells. Notably, one of the sera had high levels of HLA-C Abs (MFI 10,000–12,000) and induced considerable IgG deposition on T cells (ratio: 6; data not shown). The IgG ratio for EPCXM B cells with that particular serum was below cut-off. This may indicate that the HLA-C expression is lower on B cells than on T cells (28).

Unexpectedly, 50% of the sera from the HLA I−/II+ group resulted in EPCXM T cells IgG binding (Table 4). The IgG ratio for two of those sera (crossmatch no. 20 and 23, Table 1) were close to cut-off value which may call for re-evaluation of the IgG cut-off level. Furthermore, the patient whose sera were used in crossmatch no. 19 (IgG: 4.5, Table 1) were 3 months later found to have HLA class I Abs (highest MFI ∼4000) as determined by the more sensitive Labscreen single antigen assay. This may explain the crossmatch IgG results.

Another important observation was that 30% of the target/serum combinations that were CDC T−/B− resulted in C3d deposition on EPC. However, none of these sera were found to have HLA class I or II Abs as determined by solid-phase Ab screening. Interestingly, this data are thus in line with our previous observation that approximately 25% of all LXM− (CDC and FCXM) are EPCXM+ (manuscript submitted for publication). Importantly, this shows that EPCXM C3d assay may permit detection of complement-fixing non-HLA Abs missed by conventional LXM.

It is important to acknowledge though that the assay procedures for CDC and complement factor deposition are different with regard to target cell isolation, kinetics, read-out mode and serum source. In particular the latter will greatly influence the XM outcome. Purified rabbit complement induces lymphocytotoxicity more efficiently than human serum (data not shown; (2)). The CDC assay has been the method of choice since many years for the detection of complement-fixing anti-HLA class I and II Abs. Nevertheless, we argue that a positive CDC (in particular a positive B cell CDC) XM does not necessarily indicate a donor-specific Ab response. In this study we were not able to HLA-type the donors and thus it was not possible to check for a possible correlation between DSA and crossmatch outcome. It is generally believed that auto-Abs (both IgM and IgG) may induce false-positive B-cell XM. This highlights the importance of routinely performing autologous crossmatch tests to rule out this possibility. In future studies it

will be important to investigate whether there is a difference between the CDC and the complement factor deposition XM with regard to their capability of distinguishing between auto- and allo-Abs.

In summary, we show that the EPC crossmatch kit, XM-ONE®, can be applied for simultaneous detection of HLA class I and II IgG Ab binding and induced complement activation (C3c/d) on the surface of EPCs, T and B lymphocytes. The combined flow cytometric technique could potentially minimize the number of assays needed in the work-up prior to kidney transplantation. A potentially important advantage with the C3 assay is that the read-out, in contrast to when CDC is used, is objective and semi-quantitative. Important for the future will be to perform this flow cytometric complement activation assay with sera containing HLA and non-HLA Abs of known specificity and target cells from well-typed donors in order to fully evaluate its usefulness in a routine laboratory setting.

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Conflict of interest

JH is a part time Medical Director and board member of AbSorber AB, the company manufacturing the XM-ONE® crossmatch test. He is also a shareholder in Allenex AB, the venture capital company that is the majority owner of AbSorber AB, and his wife receives a royalty from XM-ONE® sales.

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