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The outcome of the endothelial precursor cell crossmatch test in lymphocyte crossmatch positive and negative patients evaluated for living donor kidney transplantation

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ABSTRACT

The presence of human leukocyte antigen (HLA) and non-HLA antibodies (Abs) in kidney transplant recipients is associated with graft rejections. This study reports the results of an endothelial precursor cell crossmatch (EPCXM) test for detection of non-HLA Abs and its correlation to lymphocyte crossmatch (LXM) test results, the degree and type of sensitization, and transplantation (Tx) outcome in patients evaluated for living donor (LD) kidney transplantation (KTx). Patients were tested before any pre-transplantation (pre-Tx) treatment and at Tx. Pre-Tx treatments included B cell depletion and Ab removal. Patient records were reviewed for assessment of renal graft function, results of biopsies, and identification of complications affecting the graft. Pre-Tx sera from 32% of the LD patients had IgG and/or IgM-binding donor EPCs. Twenty-five percent of the patients were EPCXM IgM+. Of the patients with negative LXM tests, 25% had EPC Abs mainly of IgM class not reactive with HLA. There was no difference in rejection frequency or serum creatinine levels between the EPCXM+ and EPCXM− groups. The pre-Tx EPCXM+ group had significantly more patients with delayed graft function. Prospective studies with appropriate control groups are needed to establish whether pre-treatments aiming at removing anti-endothelial cell antibodies, as detected by the EPCXM pre-Tx, have a beneficial effect on short-term and long-term graft survival.

1. Introduction

The clinical significance of human leukocyte antigen (HLA) antibodies (Abs) for hyperacute, acute humoral, and chronic rejection of kidney allografts has been clearly demonstrated. A number of reports have shown that Abs specific for non-HLA antigens can also cause organ allograft rejection [1,2]. The magnitude of this clinical problem is unknown but likely to be significantly smaller than the problem of HLA Abs.

The cytotoxic and flow cytometric (FC) lymphocyte crossmatch (LXM) tests are generally accepted and well suited to detect donor-specific HLA Abs (HLA DSAs) [3,4]. Recently developed and refined solid phase techniques have further improved our ability to detect and specificity-determine HLA Abs [5,6] and, thereby, to better delineate the extent of their clinical impact. Until recently, no tests were available by which a broad repertoire of Abs against non-HLA can be detected. Therefore, the clinical significance of non-HLA-specific Abs has been unclear due to the lack of suitable assays for their detection.

The fact that the vascular endothelial cells of an organ allograft are the first cells encountered by the immune cells and circulating Abs of the recipient suggests that endothelial antigens other than HLA can act as targets for rejection-mediating Abs [7]. Antigens of potential clinical importance include, but are not limited to, major histocompatibility complex (MHC) class I chain-related antigens A/B (MICA/B) [2,8] and the angiotensin II type 1 receptor [1]. Assays by which anti-endothelial cell Abs (AECAs) can be detected have in general been laborious and time-consuming, which has made them impractical for clinical use [7,9–13]. Patients who had experienced antibody-mediated rejections despite negative LXM had in high frequencies Abs against EPCs isolated from the blood of a panel of third party donors by paramagnetic beads carrying Abs specific for the Tie-2 receptor [14]. In a prospective, multicenter kidney transplantation (KTx) trial, it was shown that the presence of donor-reactive anti-EPC Abs (AEPCA), as detected by the use of a novel crossmatch (XM) kit (XM-ONE®; AbSorber AB, Stockholm, Sweden) based on this method, was strongly

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associated with acute rejections and increased serum creatinine levels at 3 and 6 months post-Tx [15].

The patients recruited into the prospective multicenter KTx study were accepted for Tx based on negative LXM tests. Because of this bias, the outcome of the EPCXM test could not be correlated to the outcome of the T and B LXM tests. However, EPCXM has been used since February 2007 as part of the immunological work-up of living donor (LD) KTx recipients at Karolinska University Hospital. In this article, we report the outcome of the EPCXM test and its correlation to the outcome of the complement-dependent cytotoxicity (CDC) and FC LXM tests, the degree and type of sensitization, and the Tx outcome in a cohort of patients evaluated for LD KTx.

2. Materials and methods

2.1. Patients

Patients included in this study ($n = 177$) were evaluated for LD KTx at Karolinska University Hospital, Huddinge, Sweden, between February 2007 and December 2009. With a few exceptions, pre-Tx testing, including HLA typing, panel-reactive Ab (PRA) assessment and HLA Ab specificity-determination, and XM tests of the patient and donor were performed 3–12 months before Tx. The FC and CDC XM tests were repeated for all patient/donor pairs just prior to Tx. The EPCXM test was repeated just prior to Tx (at-Tx testing) for those donor-recipient pairs with positive EPCXM pre-Tx testing. The at-Tx testing was done either before ($n = 11$) or after ($n = 7$) start of pre-treatment.

Of the 177 recruited patients, 100 patients were transplanted with an LD kidney between March 2007 and May 2010. Clinical data was available for 99/100 transplanted patients, and included age and gender, cause of renal failure, the immunosuppression protocol (induction and maintenance), biopsies in case of clinical signs of rejection, and serum creatinine levels at 1, 3, and 12 months post-Tx. Patients in need of dialysis post-tx were defined as having delayed graft function. The study was approved by the Central Ethical Review Board (Dnr Ö 4-2009), Stockholm, Sweden.

2.2. Immunosuppression protocols

Patients with HLA DSAs and/or with EPC Abs of IgG class based on the pre-Tx testing underwent immunoadsorption (IA) on a protein A column (Immunosorba®; Fresenius SE & Co., KGaA, Bad Homburg, Germany) or plasmapheresis (PP) prior to Tx. Patients with a positive FCXM against T cells of >40 and <160 channels shifts or a positive IgG EPCXM underwent IA. Generally, patients were transplanted if negative T FCXM or IgG EPCXM tests were obtained after treatment. Patients with EPC Abs of IgM class only were closely monitored, but did not receive any pre-treatment; nor did patients without DSAs. Patients transplanted with an ABO-incompatible kidney were treated as described elsewhere [16]. The immunosuppressive maintenance therapy was tacrolimus, mycophenolate mofetil (MMF) or azathioprine, and steroids. Except in five patients, one of whom was treated with antithymocyte globulin (ATG), two with daclizumab, and two with basiliximab, no induction therapy was given.

2.3. Genomic human leukocyte antigen typing

The HLA-A, -B, -DRβ1, and -DQβ1 loci of patients and potential donors were molecularly typed on genomic DNA using either polymerase chain reaction (PCR)-sequence-specific oligonucleotides (SSOs) (LABType®; One Lambda, Inc., Canoga Park, CA, USA) or PCR-sequence-specific primers (SSPs) (Olerup SSP®; Olerup SSP AB, Stockholm, Sweden) as described by the manufacturers.

2.4. Human leukocyte antigen antibody determination

The levels of PRAs in pre-Tx sera of patients were determined by FC analysis. Screening for anti-HLA class I and II IgG Abs using the FlowPRA® test was performed as per manufacturer's instructions (One Lambda, Inc.). In this study, patients were grouped into three categories based on their PRA reactivity: non-sensitized (<4% for both HLA class I and II), sensitized (4–80% for HLA class I and/or II), or highly sensitized (>80% for HLA class I and/or II). The HLA Ab specificity was determined using FlowPRA® Single Antigen-coated beads (One Lambda, Inc.) as described by the manufacturer. From February 2007 to April 2008, the samples were acquired on a FACScan flow cytometer and analyzed using CellQuestPro software (BD Biosciences, San Jose, CA, USA). From April 2008 onwards, samples were acquired on a Beckman Coulter FC500 (Beckman Coulter, Miami, FL, USA) and analyzed using CXP software (Beckman Coulter).

For detection of anti-HLA class I and II IgM Abs, we used Cy5-conjugated goat anti-human IgM secondary Abs (Jackson ImmunoResearch Europe Ltd., Suffolk, UK). Patient sera were considered IgM-positive when the PRA reactivity was >5% or when the histogram profile suggested reactivity with a subpopulation of the beads. Samples were acquired on a Beckman Coulter FC500 and analyzed using CXP (Beckman Coulter).

After May 2009, the level of PRA reactivity and HLA Ab specificities were determined using the Luminex-based LABScreen® PRA and Single Antigen assay, respectively (One Lambda, Inc.). In the Luminex-based assay an MFI value of >1000 above the negative control was judged positive. The LABScreen® data was treated separately from the FlowPRA® data. Furthermore, the EPCXM outcome was correlated to the HLA Ab status, as determined by the FlowPRA® assays.

2.5. Complement-dependent cytotoxicity

The standard CDC assay was performed as previously described [4]. For this purpose, T and B lymphocytes were isolated from whole blood of transplant donors by anti-CD8 and anti-CD19-conjugated magnetic beads (Invitrogen Ltd., Carlsbad, CA, USA) and were used as target cells in the CDC assay. Rabbit complement was used at a predetermined optimal dilution for cell lysis.

2.6. Flow cytometric lymphocyte crossmatch tests

Flow cytometric LXM assays, performed between February 2007 and September 2009, were performed as described elsewhere [17]. For assays performed between September 2009 and December 2009, we used peripheral blood mononuclear cells (PBMCs), prepared as target cells by gradient centrifugation. Validation demonstrated comparability between the assays (data not shown). In experiments performed after April 2008, a PE-Cy5-conjugated CD19 Ab (Beckman Coulter) was used. From February 2007 to April 2008, the samples were acquired on a FACScan cytometer (BD Biosciences) using a 256 channel linear scale. Mean channel fluorescence for patient serum that exceeded the negative control serum, with more than 10 and >20 channels for T cells and B cells, respectively, was considered positive. For XM assays performed between April 2008 and December 2009, readings were done on a Beckman Coulter FC500 flow cytometer using a 1024 linear scale. Mean channel shifts >40 and >80 for T cells and B, respectively, above negative control serum were considered positive. Inter-instrument validation demonstrated that the data generated with the instruments were interchangeable (data not shown). Samples acquired on the FACScan were analyzed using CellQuest Pro

software (BD Biosciences) and samples acquired on the FC500 were analyzed with CXP software (Beckman Coulter).

2.7. Endothelial precursor cell crossmatch assay

Endothelial progenitor cells (Tie-2$^+$ cells) were isolated with the commercially available kit XM-ONE® (Absorber AB, Stockholm, Sweden), as described by the manufacturer [15]. From February 2007 to April 2008, the samples were acquired on a FACScan cytometer (BD Biosciences) using a 1024 channel linear scale. In the second part of the study (April 2008–December 2009), we used a Beckman Coulter FC500 flow cytometer using a 1024 linear scale. Mean channel fluorescence for patient serum that exceeded the negative control serum, with >40 and >80 channels for IgG cells and IgM, respectively, was considered positive.

2.8. 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. Patient demographics

One hundred of the 177 evaluated patients were subsequently transplanted with a kidney from an LD between March 2007 and May 2010. Of the remaining patients ($n = 77$), eleven were transplanted with a kidney from a deceased donor and four with a kidney from an LD but at another Swedish Tx centre. As of June 2010, 62 patients had not yet been transplanted.

Clinical follow-up data were available until 3 months post-Tx for all but one ($n = 99$) of the patients transplanted at our centre and the demographics of those patients are shown in Table 1. In the transplanted population with follow-up data ($n = 99$), there was a significant difference in gender, rituximab pre-treatment, IA/PP, and immunosuppression protocol between the EPCXM-positive ($n = 31$) and negative ($n = 68$) (pre-Tx testing) groups (Table 1).

3.2. Overall outcome of the endothelial precursor cell crossmatch assay in patients evaluated for living donor kidney transplantation

In a non-selected group of patients ($n = 177$) undergoing evaluation for LD KTx, 199 EPCXM tests were performed between February 2007 and December 2009. Sixty-eight percent (135/199) of the

Table 1
Patient demographics.

	EPCXM pre-Tx[a]			
	All patients[b] ($n = 99$)	EPCXM+ ($n = 31$)	EPCXM− ($n = 68$)	Statistical significance[c]
Age (years)	35 ± 18	33 ± 22	36 ± 15	n.s.
Gender				
Male	60 (61%)	13 (42%)	47 (69%)	$p = 0.02$
Female	39 (39%)	18 (58%)	21 (31%)	$p = 0.02$
Original disease				
Diabetic nephropathy	8 (8%)	2 (6%)	6 (9%)	n.s.
Focal segmental glomerulosclerosis	4 (4%)	1 (3%)	3 (4%)	n.s.
IgA-nephritis	12 (12%)	2 (6%)	10 (15%)	n.s.
Polycystic kidney	12 (12%)	5 (16%)	7 (10%)	n.s.
Mesangio capillary glomerulonephritis	3 (3%)	1 (3%)	2 (3%)	n.s.
Other diseases[d]	47 (47%)	14 (45%)	33 (49%)	n.s.
Unknown etiology	13 (13%)	6 (19%)	7 (10%)	n.s.
HLA sensitization	28 (28%)	8 (26%)	20 (29%)	n.s.
Donor specific HLA Ab's (HLA DSA)	12 (12%)	5 (16%)	7 (10%)	n.s.
ABO incompatibility (AB0i)	22 (22%)	11 (35%)	11 (16%)	n.s.
Re transplant	19 (19%)	8 (26%)	11 (16%)	n.s.
Treatment pre-Tx				
Rituximab	32 (32%)	18 (58%)	14 (21%)	$p < 0.001$
AB0 spec. column (Glycosorb)	19 (19%)	9 (29%)	10 (15%)	n.s.
IA/PP[e]	10 (10%)	8 (26%)	2 (3%)	$p = 0.003$
Immunosuppression				
Tacrolimus/MMF/steroids	50 (51%)	18 (58%)	32 (47%)	n.s.
Tacrolimus/azathioprine/steroids	37 (37%)	6 (19%)	31 (46%)	$p = 0.02$
Other combinations[f]	12 (12%)	4 (13%)	8 (12%)	n.s.
Acute rejections (<3 mo post-Tx)	18 (18%)	4 (13%)	14 (21%)	n.s.
Antirejection therapy with recovery[g]	15 (15%)	3 (10%)	12 (18%)	n.s.
Antirejection therapy without recovery	2 (2%)	1 (3%)	1 (1%)	n.s.
Spontaneous recovery	1 (1%)	0 (0%)	1 (1%)	n.s.
Late rejections (>3 mo)[h]	4 (4%)	0 (0%)	4 (6%)	n.s.

[a] XM-ONE outcome for non-treated patients at pre-Tx performed 3–12 months ($N = 94$) or 2–12 days ($N = 5$) prior to Tx.
[b] Total number of patients with follow-up data transplanted at our Tx center between March 2007 and May 2010.
[c] Fisher's two-sided exact test was used throughout, except for age where the Student's *t*-test was used. $p > 0.05$ were considered non-significant (n.s.).
[d] Miscellaneous diseases such as Wegeners granulomatosis, Henoch-Schönleins purpura, kidney dysplasia, reflux nefropathy, Finnish nephrosis.
[e] Immunoadsorption (Protein A column) and/or plasmapheresis.
[f] Combinations of tacrolimus/steroids/mycophenolate mofetil (MMF) or azathioprine with other immunosuppressive agents (e.g. cyclosporin, daclizumab).
[g] Methylprednisolon therapy. In some cases in combination with rituximab and plasmapheresis.
[h] One patient experienced both an early and late rejection.

Table 2
The outcome of the EPCXM test in patients evaluated for living donor kidney transplantation.

N[b]	EPCXM[a]			
	IgG−/IgM−	IgG+/IgM−	IgG−/IgM+	IgG+/IgM+
199	135 (68%)	16 (8%)	35 (18%)	13 (7%)

[a] The number of EPCXM tests within each group: IgG−/IgM−, IgG+/IgM−, IgG−/IgM+ and IgG+/IgM+. The frequencies (%) are shown in parenthesis.
[b] The total number of EPCXM tests performed with sera from patients ($n = 177$) undergoing evaluation for living donor kidney transplantation. Note that some of the patients were cross-match tested against several donors or multiple times (>2) against a given donor. In the latter case, only the outcome from the first crossmatch is shown.

tests were negative with regard to both donor-reactive IgG and IgM Abs, while 8% (16/199) were IgG-positive, 18% (35/199) were IgM-positive, and 7% (13/199) were positive for both IgG and IgM (Table 2). Thirty-two percent (64/199) of the LD patients were therefore found to have IgG and/or IgM class Abs (HLA and/or non-HLA) directed to donor EPCs (*i.e.* Tie-2+ cells).

The presence of autoreactive AEPCAs was assessed in 19 patients with a positive EPCXM test. In 1/8 (13%) with donor-reactive IgG and in 3/11 (27%) with donor-reactive IgM, we detected auto EPC-reactive IgG and IgM, respectively (data not shown). The autoreactive sera (one IgG and three IgM) were found to be negative for HLA-specific Abs, as determined by FlowPRA® (One Lambda, Inc.) (data not shown).

3.3. Outcome of the endothelial precursor cell crossmatch assay and its correlation to the outcome of the T and B lymphocyte crossmatch tests

In parallel with the majority of EPCXM tests ($n = 160$), T and B lymphocyte CDC XM and T lymphocyte FC XM (FCXM) tests were performed (Fig. 1). In the group of patients with negative T and B CDC XM tests, 77% also lacked IgG and IgM Abs binding to donor EPCs, 5% had IgG, 15% had IgM, and 4% had both IgG and IgM binding to donor EPCs, as detected in the EPCXM (Fig. 1). In the T−/B+ CDC group of patients, the corresponding figures were 71% IgG−/IgM−, 10% IgG+/IgM−, 14% IgG−/IgM+, and 5% IgG+/IgM+, while in the T+/B+ CDC group, they were 36% IgG−/IgM−, 45% IgG+/IgM−, 0% IgG−/IgM+, and 18% IgG+/IgM+ (Fig. 1). There were significantly more positive EPCXM tests in the T+/B+ CDC XM group than in the T−/B− CDC group (7/11 vs. 25/107; $p = 0.009$), while there was no significant difference in the frequency of positive EPCXM tests between the T−/B+ and T−/B− CDC groups ($p = 0.5$). *Of* the eleven cases in the T+B+ CDC crossmatch group (of which four were both IgG and IgM negative in the EPCXM), 7/11 had HLA I and 6/11 had HLA class II IgG Abs in solid phase. Of the four patients negative in the EPCXM, all patients lacked HLA IgG Abs and consequently also donor-specific IgG Abs.

Fifteen percent (24/160) of the sera tested in the EPCXM as well as the T and B lymphocyte CDC XM were found to be positive in the T cell FCXM. In the T cell FCXM-negative group, 77% lacked donor EPC-reactive IgG and IgM Abs, 4% had IgG Abs, 15% had IgM Abs, and 4% had both IgG and IgM Abs binding donor EPCs (Fig. 1). There were significantly more positive EPCXM tests in the T cell FCXM-positive group than in the T cell FCXM-negative group (14/24 vs. 31/136; $p = 0.002$).

Of the 98 cases that had negative T and B cell CDC XM and negative T cell FCXM tests, 79% were also negative with regard to IgG and IgM Abs binding to donor EPCs, 4% had IgG Abs, 14% had IgM Abs, and 3% had both IgG and IgM Abs. Eighty-nine of the sera tested in the EPCXM were also tested with both CDC and FC with regard to the presence of donor-reactive Abs against both T and B lymphocytes, and 45 were negative in all LXM tests. Seventy-six percent of these were also negative with regard to both IgG and IgM binding to donor EPCs, 0% had only IgG Abs, 18% had IgM Abs, and 7% had both IgG and IgM Abs, as detected in the EPCXM.

3.4. Outcome of the endothelial precursor cell crossmatch assay and its correlation to the level of panel-reactive immunoglobulin G and M human leukocyte antigen class I and II antibodies

Patient sera used in 125 EPCXM tests were screened for IgG HLA class I and class II Abs by FlowPRA®. The PRA reactivity was grouped into three categories: non-sensitized (NS), sensitized (S),

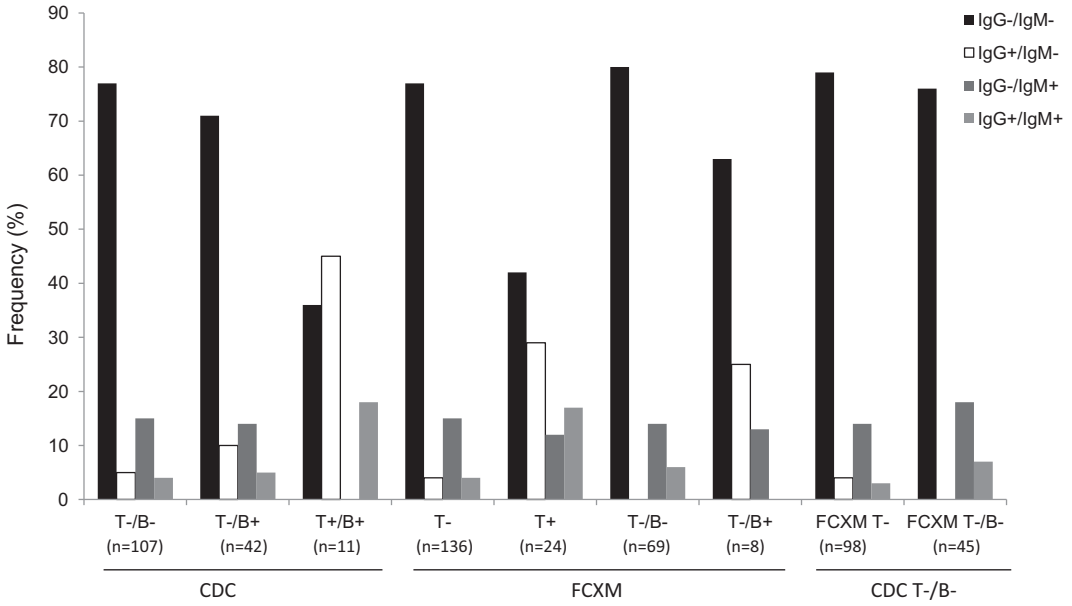

Fig. 1. The outcome of the EPCXM test in relation to the T and B lymphocyte XM outcome in patients evaluated for living donor kidney transplantation. The bars represent the percentage of EPCXM tests falling within each group: IgG−/IgM−, IgG+/IgM−, IgG−/IgM+, and IgG+/IgM+. The number of tests within each group CDC, FCXM and CDC/FCXM are shown.

Table 3
EPCXM outcome and its correlation to panel-reactive HLA class I and II IgG antibodies in patient serum.

PRA[a]	EPCXM IgG+ (n = 19)[b]	DSA+[c]	EPCXM IgG− (n = 106)	DSA+
NS	6 (32%)	ND	81 (76%)	ND
S	6 (32%)	3/6 (50%)	17 (16%)	6/17 (35%)
HS	7 (37%)	5/6[d] (80%)	8 (8%)	3/8 (38%)

[a] Groups of PRA reactivity of patient sera. NS, non-sensitized (<4% PRA, for both HLA class I and II); S, sensitized (4–80% PRA, HLA class I and/or II); HS, highly sensitized (>80% PRA, HLA class I and/or II).
[b] Number of EPCXM IgG+ or IgG− outcomes within each group of PRA reactivity. The frequencies are shown in parenthesis.
[c] Number of patients with donor-specific HLA antibodies within each group of PRA and EPCXM reactivity. The frequencies are shown in parenthesis.
[d] One donor were not HLA-typed, i.e. determination of DSA not possible.

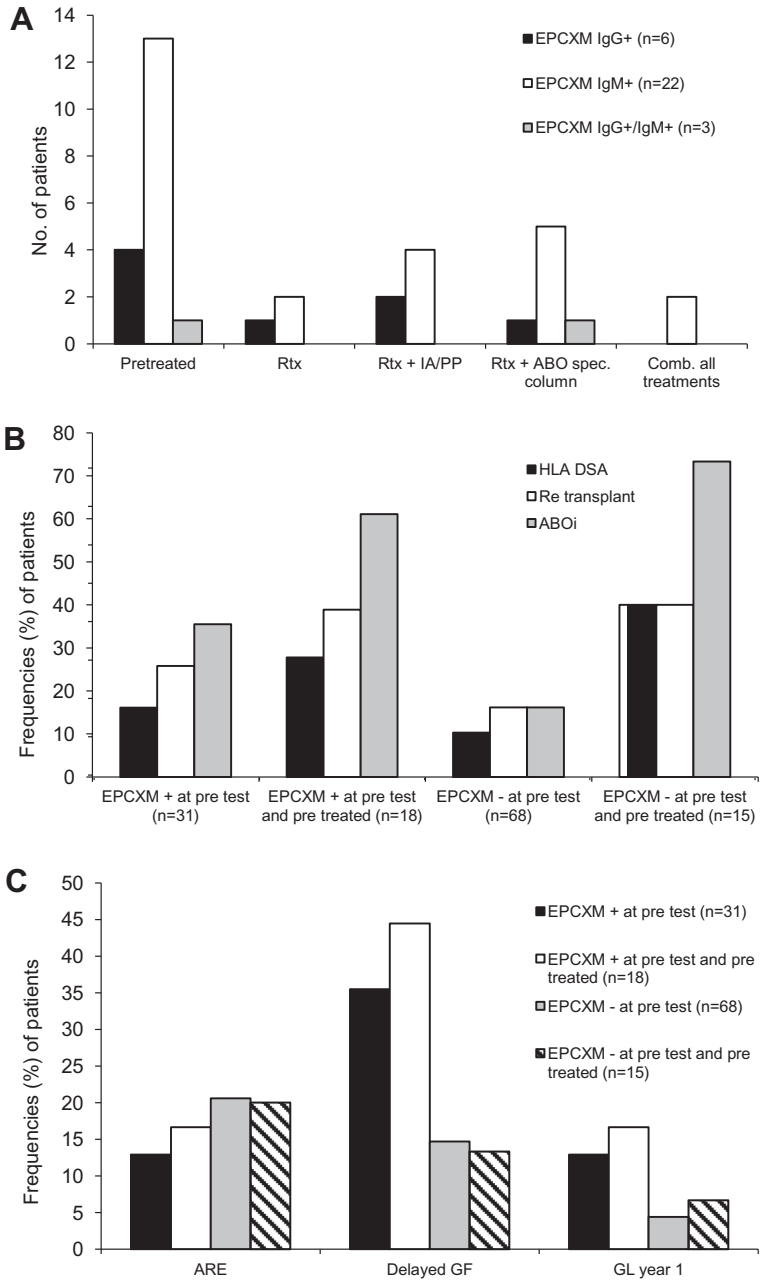

Fig. 2. (A) Distribution of pre-treatment modalities among pre-transplantation (pre-Tx) endothelial precursor cell crossmatch (EPCXM)-positive patients. Rtx = rituximab; IA/PP = immunoadsorption/plasmapheresis. (B) Frequency of patients with donor-specific HLA antibodies (HLA DSAs) receiving a re-transplant or an AB0-incompatible graft in a population of pre-transplantation (pre-Tx) endothelial precursor cell crossmatch (EPCXM)-positive and negative patients. (C) Frequency of patients with acute rejections (AREs), delayed graft function (GF), and graft loss (GL) within 1 year, among pre-transplantation (pre-Tx) endothelial precursor cell crossmatch (EPCXM)-positive and negative patients.

and highly sensitized (HS) (Table 3). Seventy percent of the LD patients were non-sensitized, 18% were sensitized, and the remaining patients (12%) were highly sensitized. Thirty-two percent of the EPCXM IgG+ patients were non-sensitized, 32% were sensitized, and 37% were highly sensitized. Seventy-six percent of EPCXM IgG− patients were non-sensitized, 16% were sensitized, and 8% were highly sensitized. There were significantly more EPCXM-positive than EPCXM-negative patients among the sensitized (6/12 vs. 17/98; $p = 0.03$) and highly sensitized group (7/13 vs. 8/89; $p = 0.0007$). For sensitized and highly sensitized patients, the HLA class I and/or class II Ab specificities were determined by single HLA antigen-coated FlowPRA® beads. In the sensitized and highly sensitized EPCXM IgG+ patient groups, 50% and 80%, respectively, had DSAs. In the sensitized and highly sensitized patients with negative IgG EPCXM, 35% and 38%, respectively, had DSAs. In patient sera with HLA class I and/or class II DSAs of IgG type, 77% (17/22) were found to have a positive T and/or B cell FCXM. A lower frequency (45%, 10/22) of DSA-positive sera resulted in an IgG-positive EPCXM (T and/or B cell FCXM vs. EPCXM; $p = 0.06$; data not shown).

The 35 sera found to have IgM Abs binding donor EPCs (see Table 2) were investigated with regard to the presence of HLA class I and class II Abs of the IgM isotype using the FlowPRA® Screening test (data not shown). Panel-reactive HLA class I and/or class II Abs of the IgM type were detected in 14% (5/35) of the EPCXM IgM+ sera, while 86% of these sera lacked HLA-specific IgM Abs. The mean percentage of IgM PRA reactivity for the sensitized patients ($n = 5$) was around 10% (data not shown).

3.5. Outcome of the endothelial precursor cell crossmatch assay and its correlation to rejection episodes and kidney function post-transplantation

Of the 99 kidney transplanted patients with follow-up data, 31 had a positive EPCXM pre-Tx (6/31 IgG+/IgM−, 22/31 IgG−/IgM+, and 3/31 IgG+/IgM+) and eleven were EPCXM-positive at Tx (1/11 IgG+/IgM−, 9/11 IgG−/IgM+, and 1/11 IgG+/IgM+). Eighteen out of 31 (58%) pre-Tx EPCXM+ patients received pre-treatment (Fig. 2A). The decision to pre-treat or not was based on, among other factors, the presence of AB0 incompatibility (AB0i) and/or DSAs. Of the pre-Tx EPCXM+ and pre-treated patients ($n = 18$), 28% had HLA DSA, 39% were re-transplanted, and 61% received an ABO-incompatible graft, while the corresponding figures for the pre-Tx EPCXM− patients ($n = 68$) were 10%, 16%, and 16% (Fig. 2B).

Eighteen of the 99 patients experienced acute rejections within 3 months post-Tx. Four patients had late rejections occurring after 3 months post-Tx (Table 1). All but one rejection were verified by biopsy. The rejection frequency in the pre-Tx EPCXM+ and pre-treated patients was 17% (3/18), while it was 21% (14/68) in the group of patients who were EPCXM− at all times (Fig. 2C). Nine percent (1/11) of the patients who were EPCXM+ at Tx experienced rejections. Thirteen of the 17 patients (76%) with rejections and a negative EPCXM at Tx were negative with regard to the lymphocyte XM tests and DSAs (data not shown). Four of the 18 patients with acute rejections had a positive FC and/or CDC LXM (22%), three of these had DSAs, and 3/18 received an ABOi kidney.

Even though there were no more rejection episodes in the pre-Tx EPCXM+ compared to the pre-Tx EPCXM− group, there were significantly more patients with delayed graft function ($p = 0.04$) in the pre-Tx EPCXM+ group (Fig. 2C). Of patients with delayed graft function 2/21 were DSA+ and 5/21 were ABOi. In many cases, the cause of delayed graft function was clearly not immunological, but in some the complication may have had an immunological component (Table 4). There was no significant association between graft loss within 1 year and the EPCXM outcome (Fig. 2C). One of seven patients with graft loss had DSA and one received an ABOi transplant.

Table 4
Reasons for delayed graft function.

	EPCXM+[a] ($n = 11$)	EPCXM− ($n = 10$)
Kidney vein thrombosis	2	2
Hemorrhage	3	1
Drainage obstruction	0	2
Circulatory disturbance	0	1
Bleedings	0	1
Kidney vein compression	1	0
Hypoperfusion	1	0
Urinary leakage	1	0
Haematoma	1	0
EBV infection	1	0
Necrosis in ureter	1	0
Unknown reasons	0	3

[a] Number of EPCXM+ and EPCXM− patients (at pre-Tx) with delayed graft function post Tx.

The highest serum creatinine levels monitored at 1 and 3 months post-Tx were found in the pre-Tx EPCXM+, DSA+ group (1 month, 147 μmol, $n = 5$; 3 months, 148 μmol, $n = 5$) (Fig. 3). The lowest levels were observed in the pre-Tx EPCXM−, DSA+ group (1 month; 83 μmol, $n = 7$; 3 months, 89 μmol, $n = 7$). However, no statistically significant difference was observed between any of the pre-Tx groups.

4. Discussion

Antibodies specific for non-HLA on donor endothelial cells can contribute to reduced graft function short- and long-term (reviewed in [18]). The extent of this clinical problem is unknown because their specificity is poorly defined and suitable assays for their detection are lacking. In the multicenter study previously mentioned, a significantly higher proportion of patients with a positive EPCXM had rejections (16/35, 46%) during the 3-month follow-up compared to those with a negative EPCXM (13/112, 12%) ($p < 0.00005$) [15]. Mean serum creatinine levels at 3 and 6 months post-Tx were also significantly higher in patients with a positive EPCXM test [15]. Based on these results and on case studies in which patients with antibody-mediated rejection (AMR) despite negative lymphocyte XM tests were shown to have Abs binding donor EPCs [19], the EPCXM was, in February 2007, included in the immunological workup of LD KTx recipients at Karolinska University Hospital.

Breimer et al. report that 24% of their patients were EPCXM+ [15]. In the LD KTx material presented here, 33% of all EPCXM tests performed were either IgG+ or IgM+, or both. The lower frequency of EPCXM+ patients in the multicenter study [15] can be explained by the fact that patients included were approved for KTx based on negative CDC, and in most cases also FC LXM tests. Further, Breimer et al. report that approximately 10% of the patients in their study had an IgM-positive EPCXM, while as many as 25% of patients in our material were IgM-positive (18% IgG−/IgM+ and 7% IgG+/IgM+) (Table 2).

Twenty-five percent of the patients ($n = 45$) negative in the conventional (CDC and FC) lymphocyte (T and B) XM tests had a positive EPCXM (18% IgG−/IgM+, 7% IgG+/IGM+). This frequency of patients with AECAs is in line with data reported by Cerilli et al., who found that 15/55 (27%) recipients of deceased donor renal allografts had a positive donor vessel-specific XM pre-Tx despite negative conventional T and B LXM tests [10]. The non-HLA targets for these Abs remain to be identified.

As expected there were more positive EPCXM tests in the LXM positive group than in the LXM negative group, because both HLA class I and HLA class II antigens are expressed on the Tie-2+ EPCs isolated with XM-ONE® [17]. However, the level of HLA class II

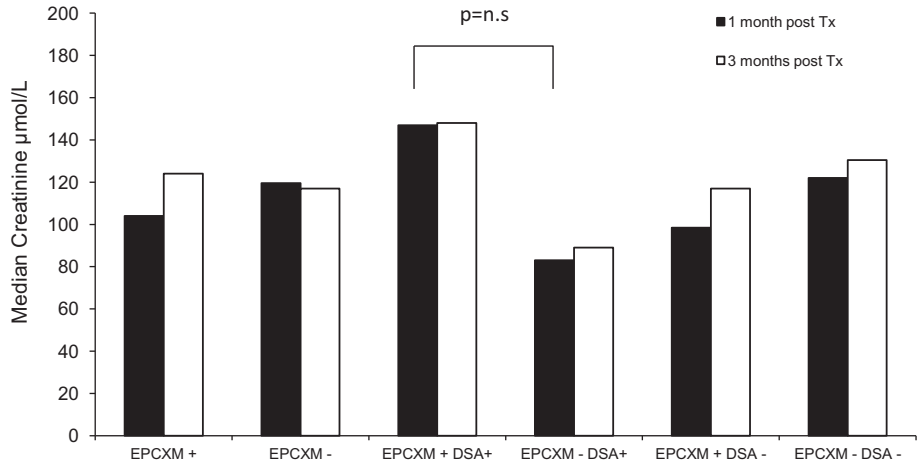

Fig. 3. Median serum creatinine levels (µmol/L) at 1 (black bars) and 3 (white bars) months in pre-transplantation (pre-Tx) endothelial precursor cell crossmatch (EPCXM)-positive and negative patients with or without donor-specific HLA antibodies (HLA DSAs).

antigen expression on EPCs is considerably lower compared to that of B lymphocytes [17]. We observed that some sera positive in the T cell FCXM were negative in the EPCXM, despite the presence of DSAs. In contrast to the HLA class II antigen expression being lower on EPCs than on B lymphocytes, the HLA class I antigen expression on EPCs and T lymphocytes is similar [17]. An explanation for the lack of reactivity in the EPCXM despite a positive T lymphocyte FCXM may be that the T FCXM with these sera was false positive (*i.e.* not due to anti-HLA class I Abs) or that the peptide repertoire, which can be expected to differ between lymphocytes and EPCs, may affect binding to the HLA+ peptide complex. The difference in the peptide repertoire may also explain why patients with HLA class I and/or class II DSA of IgG type had a significantly higher frequency of positive T and/or B cell FCXM than EPCXM tests (data not shown). Further, there were significantly more sensitized and highly sensitized patients in the EPCXM-positive than the EPCXM-negative group. Again, this can be explained by the fact that the EPCXM test detects HLA DSAs or that sensitized patients have developed Abs also against non-HLA [20].

The clinical significance of having an isolated IgM+ EPCXM is unclear. It is reasonable to assume that despite negative T and B lymphocyte CDC and negative T FCXM tests, not all patients with an isolated IgM+ EPCXM (14/98; Fig. 1) were at a higher risk of rejection. At the same time, it is important to note that the only graft loss seen in the multicenter study was in a patient with an IgM+ EPCXM [15]. In another study, a patient was reported to have had donor EPC-reactive IgM prior to Tx, despite negative lymphocyte XM tests, and to have developed AMR post-Tx [19]. Some donor EPC-reactive IgM Abs may therefore be more important than others, and finding out their specificities will be of outmost importance. Of the 35 IgM+ EPCXM-tested patients in our study, 30 lacked HLA Abs of the IgM type, suggesting that other specificities besides HLA are more likely targets for donor EPC-reactive IgM. Autologous EPCXM tests performed on a limited number of EPCXM (IgM)-positive sera showed that 27% of the allo-IgM+ EPCXM tests were positive also in the auto-IgM EPCXM. In this respect, it is worth noting that autoantigen targets for AECAs associated with decreased graft survival have been identified [21].

The frequency of patients receiving an ABO-incompatible graft, being re-transplanted, and/or having DSAs was higher in the group of patients with a positive EPCXM pre-Tx and receiving pre-treatment (rituximab alone, rituximab and IA/PP, or rituximab and antigen-specific removal of ABO Abs) compared to the group not receiving any pre-treatment or the group of patients being EPCXM− pre-Tx (Fig. 2B). Re-transplanted patients and patients with DSAs are likely to also have other Ab populations, such as those binding donor EPCs. Furthermore, having both a positive EPCXM and, for example, ABOi is likely to increase the inclination to treat, especially since the type of treatment is partly overlapping.

There was no difference in the frequency of rejections in the pre-Tx EPCXM+ and pre-Tx EPCXM− group (Fig. 2C). Whether this can be explained by the pre-treatment given to 18/31 EPCXM+ patients is not clear and prospective randomized interventional trials with proper control groups will be required to answer this question. However, there were significantly more patients with delayed graft function and even graft loss within 1 year in the pre-Tx EPCXM+ group than in the pre-Tx EPCXM− group (Fig. 2C). Even though the reason for poor graft function was clearly not immunological in many cases, in some patients the complication may have had an immunological component (Table 4). There was no statistically significant difference in creatinine levels between groups.

Further prospective studies with appropriate control groups are needed to establish whether pre-treatments aiming at removing AECAs, as detected by the EPCXM pre-Tx, have a beneficial effect on short-term and long-term graft survival.

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References

[1] Dragun D, Muller DN, Brasen JH, Fritsche L, Nieminen-Kelha M, Dechend R, et al. Angiotensin II type 1-receptor activating antibodies in renal-allograft rejection. N Engl J Med 2005;352:558.
[2] Zou Y, Stastny P, Susal C, Döhler B, Opelz G. Antibodies against MICA antigens and kidney-transplant rejection. N Engl J Med 2007;357:1293.
[3] Scornik JC. Detection of alloantibodies by flow cytometry: relevance to clinical transplantation. Cytometry 1995;22:259.
[4] Terasaki PI, McClelland JD. Microdroplet assay of human serum cytotoxins. Nature 1964;204:998.
[5] Pei R, Lee JH, Shih NJ, Chen M, Terasaki PI. Single human leukocyte antigen flow cytometry beads for accurate identification of human leukocyte antigen antibody specificities. Transplantation 2003;75:43.
[6] Zachary AA, Delaney NL, Lucas DP, Leffell MS. Characterization of HLA class I specific antibodies by ELISA using solubilized antigen targets: I. Evaluation of the GTI QuikID assay and analysis of antibody patterns. Hum Immunol 2001;62:228.
[7] Glotz D, Lucchiari N, Pegaz-Fiornet B, Suberbielle-Boissel C. Endothelial cells as targets of allograft rejection. Transplantation 2006;82(Suppl. 1):S19.

[8] Terasaki PI, Ozawa M, Castro R. Four-year follow-up of a prospective trial of HLA and MICA antibodies on kidney graft survival. Am J Transplant 2007;7:408.
[9] Cerilli J, Bay W, Brasile L. The significance of the monocyte crossmatch in recipients of living-related HLA identical kidney grafts. Hum Immunol 1983;7:45.
[10] Cerilli J, Clarke J, Doolin T, Cerilli G, Brasile L. The significance of a donor-specific vessel crossmatch in renal transplantation. Transplantation 1988;46:359.
[11] Le Bas-Bernardet S, Hourmant M, Coupel S, Bignon JD, Soulillou JP, Charreau B. Non-HLA-type endothelial cell reactive alloantibodies in pre-transplant sera of kidney recipients trigger apoptosis. Am J Transplant 2003;3:167.
[12] Moraes JR, Luo Y, Moraes ME, Stastny P. Clinical relevance of antibodies to non-HLA antigens in organ transplantation. Clin Lab Med 1991;11:621.
[13] Sumitran-Karuppan S, Tyden G, Reinholt F, Berg U, Moller E. Hyperacute rejections of two consecutive renal allografts and early loss of the third transplant caused by non-HLA antibodies specific for endothelial cells. Transpl Immunol 1997;5:321.
[14] Vermehren D, Sumitran-Holgersson S. Isolation of precursor endothelial cells from peripheral blood for donor-specific crossmatching before organ transplantation. Transplantation 2002;74:1479.
[15] Breimer ME, Rydberg L, Jackson AM, Lucas DP, Zachary AA, Melancon JK, et al. Multicenter evaluation of a novel endothelial cell crossmatch test in kidney transplantation. Transplantation 2009;87:549.
[16] Tyden G, Kumlien G, Genberg H, Sandberg J, Lundgren T, Fehrman I. ABO incompatible kidney transplantations without splenectomy, using antigen-specific immunoadsorption and rituximab. Am J Transplant 2005;5:145.
[17] Alheim M, Johansson SM, Hauzenberger D, Grufman P, Holgersson J. A flow cytometric crossmatch test for simultaneous detection of antibodies against donor lymphocytes and endothelial precursor cells. Tissue Antigens 2010;75:269.
[18] Sumitran-Holgersson S, Holgersson J. Clinical importance of non-HLA antibodies in solid organ transplantation. Curr Opin Organ Transplant 2006;11:425.
[19] Holgersson J, Elsheikh E, Grufman P, Sumitran-Holgersson S, Tydén G. A case of acute vascular rejection caused by endothelial-reactive non-HLA antibodies. Clin Transpl 2007;2006:535.
[20] Opelz G. Non-HLA transplantation immunity revealed by lymphocytotoxic antibodies. Lancet 2005;365:1570.
[21] Mahesh B, Leong HS, McCormack A, Sarathchandra P, Holder A, Rose ML. Autoantibodies to vimentin cause accelerated rejection of cardiac allografts. Am J Pathol 2007;170:1415.