Open Access

OBM Transplantation

LIDSEN Publishing Inc.

Original Research

Detection of Complement-Fixing Anti-HLA Antibodies by C3d-Detecting Luminex Technique. Is There Any Additional Diagnostic Value Not Available Through the Use of Standard Single Antigen Antibody Specification?

Anastasia Doroshenko [1], Matthias Reimers [2], Gary Sawers [3], Gerald Schlaf [1, *]

1. Tissue Typing Laboratory (GHATT), University Hospital Halle/Saale, Ernst-Grube-Straße 40, 06120 Halle/Saale, Germany; E-Mails: anastasia.doroshenko@uk-halle.de; gerald.schlaf@uk-halle.de
2. Institute for Tumor Immunology, Martin-Luther University Halle-Wittenberg, Halle/Saale, Germany; E-Mail: Matthias.Reimers@uk-halle.de
3. Institute of Biology/Microbiology, Martin-Luther University Halle-Wittenberg, Halle/Saale, Germany; E-Mail: gary.sawers@mikrobiologie.uni-halle.de

* **Correspondence:** Gerald Schlaf; E-Mail: gerald.schlaf@uk-halle.de

Academic Editor: Vikash Mishra

Special Issue: HLA in Transplantation: Typing, Matching and Outcomes

OBM Transplantation
2026, volume 10, issue 3
doi:10.21926/obm.transplant.2603277

Received: April 23, 2026
Accepted: September 15, 2026
Published: September 22, 2026

Abstract

Donor-specific antibodies (DSA) directed against human leukocyte antigens (HLA) are regarded as the principal cause of graft failure, including allograft loss. Especially cytotoxic, i.e., complement-fixing, antibodies have long been classified as clinically highly deleterious in the context of graft failure. The complement-dependent cytotoxicity assay (CDC), performed in cell-tray analyses, has been used to define these antibodies for more than 40 years, but generally lacks sensitivity. Due to its limited resolution, which does not exceed the single-donor level, it also cannot specify distinct antigens, especially in highly immunized patients. About fifteen years ago, this problem was overcome by broadly established Luminex-based single-antigen specification assays (SAB), which identify antibodies directed against recombinant single HLA antigens. To specify complement-fixing antibodies of postulated

higher clinical relevance, these assays were supplemented by two commercial kits, initially by the C1q assay and later by the C3d assay. Here, we used the C3d assay to investigate serum from 74 selected patients on kidney waiting lists, which represent three groups, each with increasing levels of immunization/panel-reactive antibodies (%PRA): (i) with only anti-HLA class I antibodies (n = 24); (ii) with only anti-HLA class II antibodies (n = 21); and (iii) with combined anti-HLA class I and class II antibodies (n = 29). Our data show that C3d assay-defined cytotoxicity, especially for anti-HLA class I antibodies, strongly correlates with the mean fluorescence intensity (MFI). Anti-HLA class I antibodies appear cytotoxic when corresponding SAB MFI values exceed 6,000 to 7,000, whereas below this threshold cytotoxicity does not appear. Dilution experiments showed that cytotoxic antibodies could be converted to non-cytotoxic antibodies simply by falling below this threshold MFI value. In contrast, anti-HLA class II antibodies with cytotoxicity-defining threshold MFI-values between 1,500 and 2,000 differ by having a higher proportion of non-cytotoxic antibodies that even lie above these MFI-values. Nevertheless, we conclude that due to strong correlations with SAB MFI-values (Pearson correlation coefficients of 0.69 for HLA-class I and 0.63 for HLA-class II), there is insufficient clinical applicability to justify the considerable amount of work involved, and especially the high associated costs, for routine use of the C3d assay.

Keywords
Antibody-specification; anti-HLA antibodies; C3d; donor-specific antibodies (DSA); kidney allografting; Luminex technique; mean fluorescence intensity (MFI)

1. Introduction

More than 50 years ago, the correlation between antibodies directed against donor tissue antigens and hyperacute allograft rejection was first described [1]. Later studies provided evidence that these donor-specific antibodies (DSA) are primarily directed against the major histocompatibility complex (MHC), the human leukocyte antigens (HLA) [2, 3]. Based on these findings, and in order to prevent prospective kidney recipients from hyperacute or acute allograft rejections, a complement-dependent cytotoxicity crossmatch technique (CDC-XM) was developed and established as the standard technique in the 1970s. Lymphocytes isolated from a given donor's blood are incubated with the prospective recipient's serum to facilitate a complement-dependent attack after adding complement from rabbits. Since the development of the technique, a resulting DSA-mediated cytotoxic (complement-dependent) effect, i.e., a positive outcome of this CDC-XM, is classified as a clear contraindication defining the incompatibility of this contemplated donation. This is because initiation of the classical pathway of complement activation through DSA is regarded as the most prominent mechanism provoking allograft injury. Observations made in the early 1990s, which indicated that cleavage products of the complement activation cascade, such as C3d and C4d, were immunohistochemically detectable in the capillaries of the majority of rejected kidney allografts were in accord with this original functional assumption [4-7].

This CDC-XM technique was also implemented as an antibody specification assay. This assay, which is referred to as the 'cell tray analysis', uses a cell panel of PBL or CLL and represented the

'gold standard' for specifying anti-HLA antibodies until the late 1990s. However, this CDC-based assay was characterized by severe drawbacks, including limited sensitivity and especially its specificity, mainly due to its poor level of resolution [8, 9]. Furthermore, the CDC-XM, as well as the cell tray analyses, were highly susceptible to false-positive results when sera were used from recipients who suffered from autoimmune diseases, particularly of the immune complex type (type III) [10]. Additionally, using PBL-based cell trays, it was not possible to define unequivocally antibodies directed against HLA-class II antigens, which severely limited the procedure. Nevertheless, due to logistical problems or inadequate storage conditions, the background signal caused by dead cells frequently reduced the capability of identifying the antibody-mediated reactions.

In order to circumvent these issues, various solid-phase-based assays were developed and implemented in many tissue-typing laboratories as early as the 1990s, such as the flow cytometric FlowPRA assay, which in its initial design comprised a total of 60 HLA class I or class II specificities. These were detectable as panels of either HLA-class I or class II antigens on the surface of microparticles/beads, which provided a platform for antibody specification that was independent of cell vitality [11, 12]. Although FlowPRA cytometric analysis represented the most sensitive method among the techniques utilized, it was characterized by a high technical complexity and a high financial investment.

An alternative approach used ELISA-based screening and antibody-specification assays, which had lower technical complexity and had lower associated costs [13-16]. A further advantage of this technique was that most laboratories had an ELISA reader as standard equipment.

In terms of both sensitivity and specificity, nearly all of these solid-phase assays were superior to the original cell tray analyses. However, the resolution of the solid-phase assays, with only a few exceptions, did not attain the level to define specificities, which are directed against single antigens [9]. It should be noted that for highly immunized patients in particular, a high level of resolution is indispensable for the implementation of "virtual cross-matching", denoting the concept of validly excluding organ donations as a consequence of a recipient's identified antibody specificities, without the approach of physical (*de facto*) cross-matching. Apart from the development of the DynaChip technology as the only completely automated system, which was later discontinued by the manufacturer for commercial reasons, only the Luminex multiplex bead-based technology remains as a system with the capability to analyse simultaneously up to 100 microparticles carrying different single antigens [10, 17, 18]. Although the DynaChip array has been re-launched as HISTO SPOT® HLA AB, a single-antigen assay by another manufacturer [19], the Luminex technique remains to provide the application of single-antigen specification assays. Two manufacturers supply it: Thermo Fisher/One Lambda and Werfen/Immucor. Nevertheless, the Luminex-based arrays, especially the LABScreen Single Antigen HLA class I and II supplied by Thermofisher/One Lambda, have been discussed controversially in the context of the validity and clinical relevance of some data. False-positive results have been reported for subjects without any allo-immunization due to 'natural' antibodies, which are directed solely against recombinantly generated, i.e., denatured, and not against physiologically assembled HLA molecules [20-26]. Beyond this aspect of allograft-irrelevant natural antibodies, a second problem concerns low-titer antibodies, which are characterized by low MFI values. For several years, it has been discussed whether all DSA, which are identifiable in the Luminex Single Antigen Technique, are of clinical relevance. Moreover, this is important to define the MFI threshold value, above which DSA should be regarded as clinically relevant [27, 28]. False-

negative results have additionally and repeatedly been reported using the Luminex technique, mainly as a consequence of the prozone effect [29, 30]. This indicates that any methodical standard necessary to define true from artificial results has not yet been defined due to various reasons, including the different technical approaches adopted [9, 19], as well as significant differences in the outcome of Luminex-based single-antigen assays manufactured by the two suppliers [9, 26, 31].

Given the published and widely recognized clinical significance of complement-fixing antibodies and the manifold drawbacks of cell-tray analyses for reliable identification, supplementary Luminex-based complement-activation assays (C1q/One Lambda-Thermofisher and C3d/Werfen-Immucor) have been developed. In addition to allowing specification of these antibodies, these assays provide evidence suggesting that these antibodies include activation of the complement cascade. In contrast to hitherto unresolved questions resulting from the diverse Luminex-based specification assays, the *in vitro* assessment described in this current study aimed to reliably identify the antibodies responsible for the deleterious activation of the complement system. We present data for the Werfen/Immucor C3d-solid-phase assay based on analysis of 74 selected sera from patients on a kidney waiting list. This cohort represents three groups of patients with increasing levels of immunization/panel-reactive antibodies (%PRA). These groups include patients exhibiting: (i) anti-HLA class I antibodies only (n = 24), with 6-92% PRA; (ii) anti-HLA class II antibodies only (n = 21), with 10-97% PRA; and (iii) combined anti-HLA class I and class II antibodies (n = 29), with 0-98% PRA.

2. Patients and Methods

2.1 Patients

Sera of 74 patients from the kidney waiting list were collected between March and November 2024 and retrospectively analyzed. Prior to analysis, the sera were stored at -28°C. The sera of the patients represented three groups, which could be analyzed separately. The first group comprised patients (n = 24) exhibiting only anti-HLA-class I antibodies; the second group included patients (n = 21) exhibiting only anti-HLA-class II antibodies; whereas the patients of the third group (n = 29) were characterized by antibodies against HLA-class I as well as class II antigens. All groups were characterized based on the Luminex-based single donor-defined levels of HLA-specific immunization of the patients, which lay between either no or low levels (0-10%, respectively) or high levels (92-98%). All serum samples were first analyzed for anti-HLA antibodies using either the classic, i.e., CDC-based cell-tray analysis, or the Luminex-based antibody specificity at the corresponding single-donor (SD) level. Increasing PRA values and corresponding antibody specificities, as comparatively determined by Luminex-based single-donor analyses and the corresponding CDC-based cell-tray analyses, are presented in Table 1. Furthermore, the sera of all patients were investigated using Luminex-based antibody specification at the single-antigen (SA) level and, additionally, all antibodies directed against definable specificities were tested for their additional capability of activating the complement cascade through the use of the C3d detection kit.

Table 1 Increasing Luminex Single-Donor (ID-) based PRA values and identifiable antibody specificities in comparison with the corresponding data of CDC-based cell-tray analyses.

Pat. ID	PRA [%] Lum-ID		Spec. Lum-ID (n)	PRA [%] CDC	Spec. CDC (n)
	Cl. I	Cl. II		-DTT	
5	6	0	1	15	0
6	8	0	1	8	0
18	8	0	1	19	0
19	8	0	2	23	9
1	10	0	1	12	1
4	14	0	2	18	0
21	14	0	1	18	0
11	18	0	3	34	1
15	20	0	3	5	0
2	24	0	2	17	0
14	30	0	3	12	0
22	32	0	6	12	0
10	34	0	5	30	0
23	44	0	6	17	0
9	46	0	5	25	2
17	48	0	9	8	0
16	54	0	15	17	1
3	60	0	13	27	0
20	60	0	2	19	0
8	62	0	8	30	5
13	66	0	13	7	0
24	68	0	17	38	6
7	86	0	19	68	7
25	92	0	15	17	1
55	0	10	2	n.def.	0
57	0	10	1	n.def.	0
58	0	10	1	n.def.	0
59	0	12	2	n.def.	0
60	0	12	1	n.def.	0
61	0	12	2	n.def.	0
62	0	13	1	n.def.	0
63	0	13	1	n.def.	0
64	0	14	3	n.def.	0
65	0	19	4	n.def.	0
66	0	19	3	n.def.	0
67	0	24	5	n.def.	0
68	0	29	2	n.def.	0
69	0	30	1	n.def.	0

70	0	47	4	n.def.	0
71	0	48	5	n.def.	0
72	0	50	5	n.def.	0
73	0	52	2	n.def.	0
74	0	57	4	n.def.	0
75	0	67	8	n.def.	0
56	0	97	14	n.def.	0
50	0	0	0	100	0
52	1	0	1	92	0
54	2	8	1	18	0
27	8	3	1	30	0
28	10	13	4	35	0
42	10	27	1	38	0
43	10	37	2	27	0
49	10	4	0	39	0
34	10	93	36	97	0
51	12	0	1	87	0
30	14	10	3	22	0
33	16	33	2	20	0
36	16	24	6	34	0
44	16	45	4	28	0
29	18	67	9	57	0
38	20	60	3	43	0
46	20	64	8	22	0
26	24	10	2	15	0
53	24	17	2	n.d.	n.d.
41	26	5	3	23	0
32	28	33	7	37	0
37	30	43	7	33	1
35	44	10	12	35	1
45	50	10	11	45	2
47	72	13	11	53	1
40	80	63	26	47	5
31	82	97	35	30	1
39	88	10	14	38	2
48	98	77	26	83	5

Blue, patients exhibiting only anti-HLA class I antibodies; Green, patients exhibiting only anti-HLA class II antibodies; Yellow, patients exhibiting both anti-HLA class I and class II antibodies; bold and underlined lettering, high CDC-based values due to underlying autoimmune phenomena without corresponding Luminex-definable specificities.

CDC, Complement-Dependent-Cytotoxicity/Lymphocytotoxicity Assay (Cell-Tray Analysis); Lum-ID, Luminex-based Single-Donor (ID-) assay; n.d., not determined due to lack of serum; n.def., not definable; PRA [%], panel-reactive antibodies [%]; Spec., antibody specificities.

2.2 Luminex-Based Werfen/Immucor Antibody Specifications at the Single-Donor (SD/ID) and the Higher Single-Antigen (SA) Levels

2.2.1 Luminex Single-Donor (SD/ID) Analysis

Generally, Luminex-based antibody specification at the SD-level of resolution was performed to provide a level of resolution comparable to that of CDC-based cell-tray analysis. While the data from high-level single-antigen specifications differ from those of lower-level cell-tray assays, it was meaningful to compare solid-phase-based antibody specifications with cell-tray analysis at the corresponding levels of resolution. Single-donor analysis was performed at room temperature in accordance with the guidelines provided by the manufacturer (Werfen/Immucor, Stamford, USA). Briefly, 12.5 µl of each serum sample and 12.5 µl each of a positive and a negative control serum were mixed with 40 µl of washing buffer using the wells of a 96-well plate. A 5 µl aliquot of thoroughly resuspended class I or class II beads was added, followed by an incubation in the dark for 30 min using an incubation shaker. After three additional washing steps using 200 µl of washing buffer for each step, 50 µl of secondary phycoerythrin-conjugated anti-human IgG antibody was added. Incubation took place in the dark, as described above. The beads in each well were thoroughly resuspended using 150 µl of washing buffer and afterwards analyzed within one hour using the Luminex 200 system. Three beads representing the negative controls Con1-3 (Con1 coated with albumin, Con2 coated with glycoprotein IV, Con3 uncoated blank beads) should exhibit low MFI values, as defined for each lot by the manufacturer. Beads were calculated to be either positive or negative using the standard evaluation protocol of the Match IT software of Werfen/Immucor.

2.2.2 Luminex Single-Antigen Analysis (LSA)

The procedure was performed at room temperature and in complete accordance with the instructions provided by the manufacturer Werfen/Immucor (Lifecodes SA class I & II single antigen assays, Stamford, USA). Briefly, 10 µl of each serum sample and, additionally, 10 µl of positive and negative control serum were mixed with 40 µl of Luminex Single Antigen (LSA) class I or class II beads. Incubation took place in the dark for 30 min. After washing, a 50 µl aliquot of secondary phycoerythrin-conjugated anti-human IgG antibody was added, followed by an incubation for 30 min in the dark. Afterwards, 150 µl of washing buffer was added per well. The beads were thoroughly resuspended, and the samples were measured using the Luminex 200 system. Data analysis was performed according to the standard evaluation protocol using the Match IT software of Werfen/Immucor, setting an overall lower cut-off value of 750. In order to avoid lot-to-lot variations, all analyses were carried out using kits from a single lot.

2.2.3 C3d-Binding Assay (C3d)

The same sera tested for specifiable anti-HLA antibodies were subsequently tested for their complement-activating capability, as illustrated by the flow scheme of Figure 1. Again, all procedures were performed at room temperature in complete accordance with the manufacturer's instructions (Werfen/Immucor Lifecodes C3d assay, Stamford, USA). Generally, the same antigen-coated beads used for antibody specification were used for detecting their complement-activating capacity. Briefly, 1 µl of C3d-positive control bead suspension was incubated with 40 µl of HLA class

I or class II single-antigen bead suspension and subsequently mixed with a 10 µl aliquot of serum from the respective patients (alternatively, samples were mixed with 10 µl of positive or negative control serum, respectively). After an incubation period of 30 min in the dark, 30 µl of serum (from male and healthy donors) was added as a source of complement, and the mixture was incubated in the dark for a further 30 min. After three washing steps, 50 µl of monoclonal phycoerythrin-conjugated anti-human C3d antibody was added, and the suspension was incubated for a further 30 min in the dark. After two final washing steps, the bead suspension was thoroughly resuspended and analyzed using the Luminex 200 analyzer. Positive results for C3d were automatically assigned by the xPonent software when two of the following three criteria were fulfilled: (i) background-adjusted MFI value >1,500; (ii) R-strength >4.0; and (iii) BCR-neg >4.0. The procedure is illustrated in Figure 1.

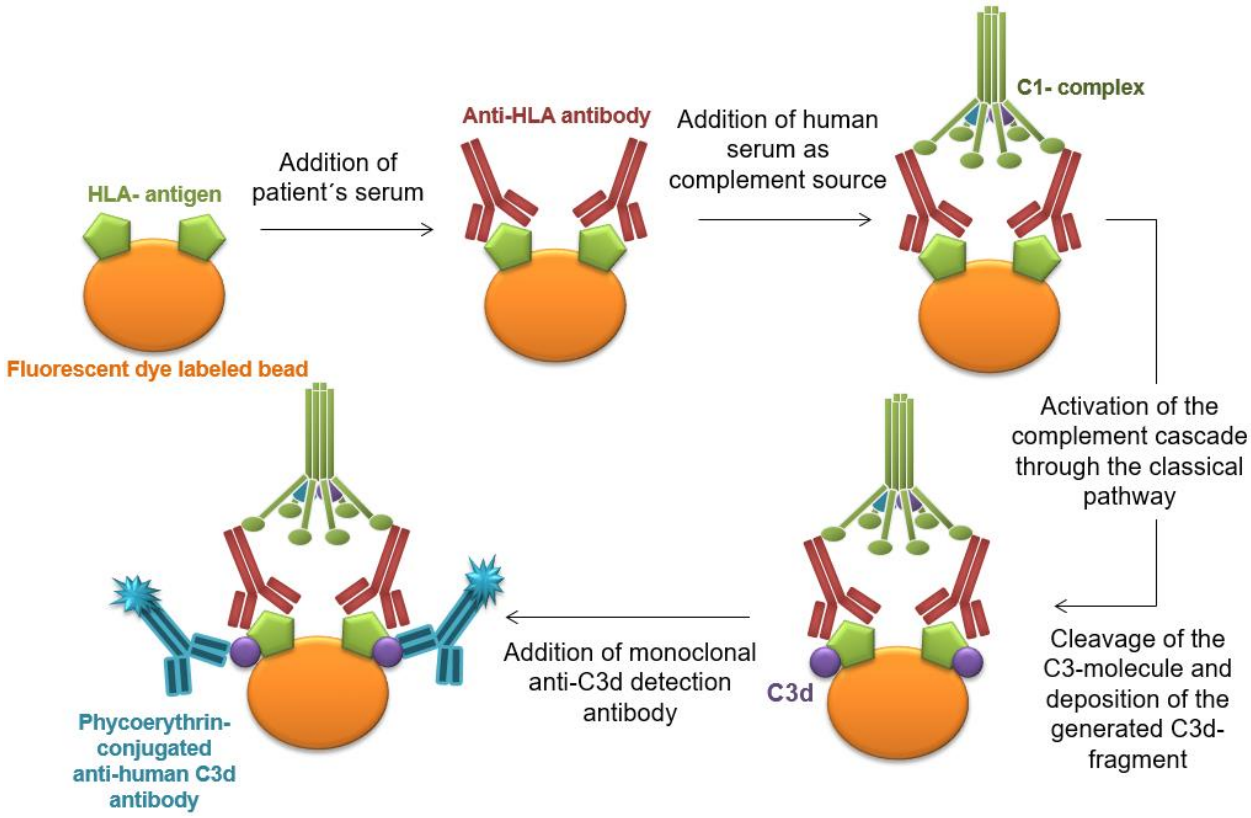

Figure 1 Workflow of the C3d assay identifying complement-fixing antibodies.

2.3 Cell-Tray Analysis (CDC-Based Antibody Specification Using a Panel of 60 PBL Cells)

Commercial, deep-frozen (-80°C) SeraScreen FCT 60 cell trays (BAG, Lich, Germany) were thawed for 5 min directly before use at room temperature (RT). Cells were washed with 10 µl of FCS (25% v/v)/PBS washing buffer by incubating them for 10 min at RT. The washing buffer was removed, and 2 µl of patient serum was added to each well and incubated for 30 min at RT. Afterwards, a 5 µl aliquot of rabbit complement was added to each well to induce complement-mediated cell lysis. The incubation took place for 60 minutes. Finally, a 2 µl aliquot of Fluoroquench solution (BmT/One Lambda, Meerbusch, Germany), containing both acridine orange (vital dye) and ethidium bromide (lethal dye), was added to each well and incubated for another 15 min in the dark. We evaluated the percentage of red/dead cells using the National Institutes of Health (NIH) scoring system.

Reactions characterized by at least an additional 20% of dead cells (NIH score of 4) in comparison to the negative control serum (score 1) were regarded as positive. Positive control sera had to exhibit an NIH score of 8 (at least 80% of dead cells) to define the results of the serum under investigation as valid. Furthermore, cell vitality had to attain at least 90% (not exceeding a score of 1), as demonstrated through the use of the negative control serum. Panel-reactive antibodies (PRA) were calculated as follows: PRA [%] = number of positive reactions/number of wells (60) × 100.

3. Results

3.1 Superiority of the Luminex-Based Specification Over the Cell-Tray Analysis

Patients were initially chosen in terms of increasing panel-reactive antibodies (PRA%) as summarized in Table 1. We first compared the data of Luminex-based antibody specifications of the single-donor (SD/ID) level with those of PBL-based cell-tray analyses in order to define a comparable level of panel-reactive antibodies and to determine clearly definable antibody specificities for both systems (Table 1).

The superiority of Luminex-based specifications was evident particularly for the group of patients (green in Table 1) who exhibited only anti-HLA class II antibodies. No anti-HLA class II antibodies were unequivocally identifiable for all of these patients, because at most 25% of PBL, representing the portion of antigen-presenting cells, led to NIH scores not higher than 2/4 (doubtful to weakly positive). Thus, for all patients of this group (green in Table 1) the PRA-values as well as antibody specificities were not validly identifiable. It should be noted in this context that cell trays plated with chronic lymphocytic leukemia (CLL) cells bearing HLA class II antigens were no longer commercially available when these investigations were carried out, leading to a limitation in terms of identifying anti-HLA class II antibodies. The same problem occurred for patients exhibiting anti-HLA class I as well as anti-HLA class II antibodies (yellow in Table 1), which are listed by increasing percentages of HLA-class I-specific antibodies. All CDC-based specificities clearly define antibodies that are directed against HLA-class I antigens (Pat. ID 31, 35, 37, 39, 40, 45, 47 and 48 in Table 1), whereas no HLA-class II specificities were definable. Thus, again the cell-tray-based specification data provided in this group are, without exception, based on anti-HLA class I antibodies. Luminex-based solid-phase assays allowed valid antibody specifications in 72/74 cases (97%), in contrast to only 17/74 cases (23%) of cell-tray analyses. Of interest are three patients (Pat. ID 50, 51, 52) that exhibited CDC-based PRA values of 100%, 87%, and 92%, respectively. These three patients (highlighted by bold and underlined lettering in Table 1) were characterized by underlying immune-complex (type III) autoimmune diseases leading to nonspecific cell-tray signals, as opposed to the absence of HLA specificities (Pat. ID 50), or exhibiting only one specificity (Pat. ID 51, 52) validly identifiable by Luminex analyses. Patient ID 50 was the most prominent example, who, although exhibiting scores of between 6 and 8 in all wells of the cell-trays, showed no definable specificity in the Luminex-based assay. Generally, and as anticipated, the superiority of the Luminex-based analysis over CDC-based cell-tray analysis is clearly demonstrable, despite the Luminex-based technique not being implemented at the highest single-antigen level of resolution, but instead at the lower SD-level. This was done to make both procedures comparable. In this context, it must be critically assessed whether the 13 or more antibody specificities, as defined by Luminex-based single-donor analysis (e.g. Pat. ID 3, 7, 13, 16, 24, 25), really represent validly definable specificities and are not in part due to misinterpretation of the data. One drawback of using the SD-system alone is its limited

resolution, because overlapping single antigens result from immobilizing HLA-class I or class II antigens from a single donor on a single carrier bead. Thus, multiple specificities may be better defined using high-level single-antigen resolution. Taken together, the results show significant deficiencies in the cell-tray analyses and point to the need to establish a solid-phase-based procedure in order to identify complement-activating antibodies.

3.2 Comparative Antibody Specifications Using the Luminex Single-Antigen Specification Kit and the Additional C3d Module to Classify These Antibodies in Terms of Complement Activation

The Luminex Single-Antigen (LSA) antibody specification system allows antibody specification at the highest level of resolution, as only single antigens are immobilized on individual beads after their recombinant production. The additional C3d module allows the classification of the specified antibodies as either complement-activating or not. Typical data are shown for patient ID 8 who, in contrast to the single-donor level with eight specificities (Table 1), exhibited 14 specificities at this higher level of resolution with immobilized single alleles (Figure 2a).

a

Pat. ID	LSA molecular/allelic specificities	LSA corresponding serological equivalents	LSA-C3d allelic specificities of complement-fixing Ab	LSA-C3d serological equivalents of complement-fixing Ab
8	A*01:01	A1	A*01:01	A1
	A*23:01	A23(9)	A*23:01	A23(9)
	A*24:02	A24(9)	A*24:02	A24(9)
	A*26:01	A26(10)	A*26:01	A26(10)
	A*29:01	A29(19)	-	-
	A*29:02	A29(19)	A*29:02	A29(19)
	A*36:01	A36	A*36:01	A36
	A*43:01	A43	A*43:01	A43
	A*80:01	A80	A*80:01	A80
	B*15:12	B76(15)	B*15:12	B76(15)
	B*44:02	B44(12)	-	-
	B*44:03	B44(12)	B*44:03	B44(12)
	B*45:01	B45(12)	-	-
	B*82:02	B82	B*82:02	B82

b

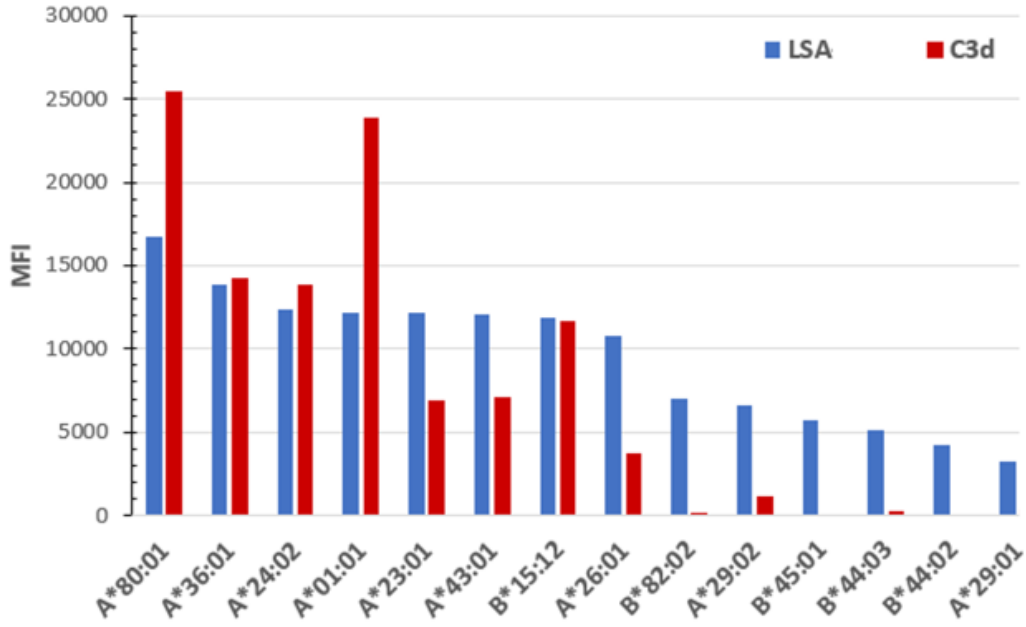

Figure 2 (a) Listing of the complete number of allelic HLA-class I antibody specificities of patient ID 8 using the Luminex-based single antigen specification (LSA, column I) and their serological equivalents (LSA, column II) in comparison to the specificities of complement-fixing antibodies (green lettering) shown using the allelic notation (C3d, column III) and by the serological equivalents (C3d, column IV). (b) MFI values of the Luminex-based allele-specific antibodies (blue histograms, LSA) in comparison with corresponding signals generated by detecting the complement cleavage product C3d (red histograms, C3d).

The 14 molecular/allelic specificities depicted in column 1 were reducible to 12 serological specificities/phenotypes, which are depicted in column 2 of Figure 2a. Thus, four additional serological specificities (HLA-A43, B45, B76 and B82) were identifiable in comparison to the specification performed at the SD level. Of the 14 allelic specificities identified, only three (A*29:01, B*44:02 and B*45:01) were identified to lack the activation of the complement system, whereas 11 were shown to be capable of binding complement. There is a clear correlation between the MFI values of the identified antibody specificities (blue histograms) and the C3d-specific signal (red histograms), as an indicator of the complement-fixing capacity of the respective antibodies. All antibodies directed against their specificities with MFI-values higher than 6,000 exhibited complement-fixing capacity, whereas antibodies with MFI-values lower than this threshold nearly (B*44:03), or completely (B*45:01, B*44:02 and A*29:01), lacked this feature (Figure 2b). In order to substantiate this assumed correlation, the antigen-specific MFI values of patient ID 8 were plotted against the corresponding C3d-specific MFI values. These results summarize those of all 74 patients. Figure 3 shows their MFI values separated by HLA-class I (orange squares) and class II (blue circles) specific antibodies.

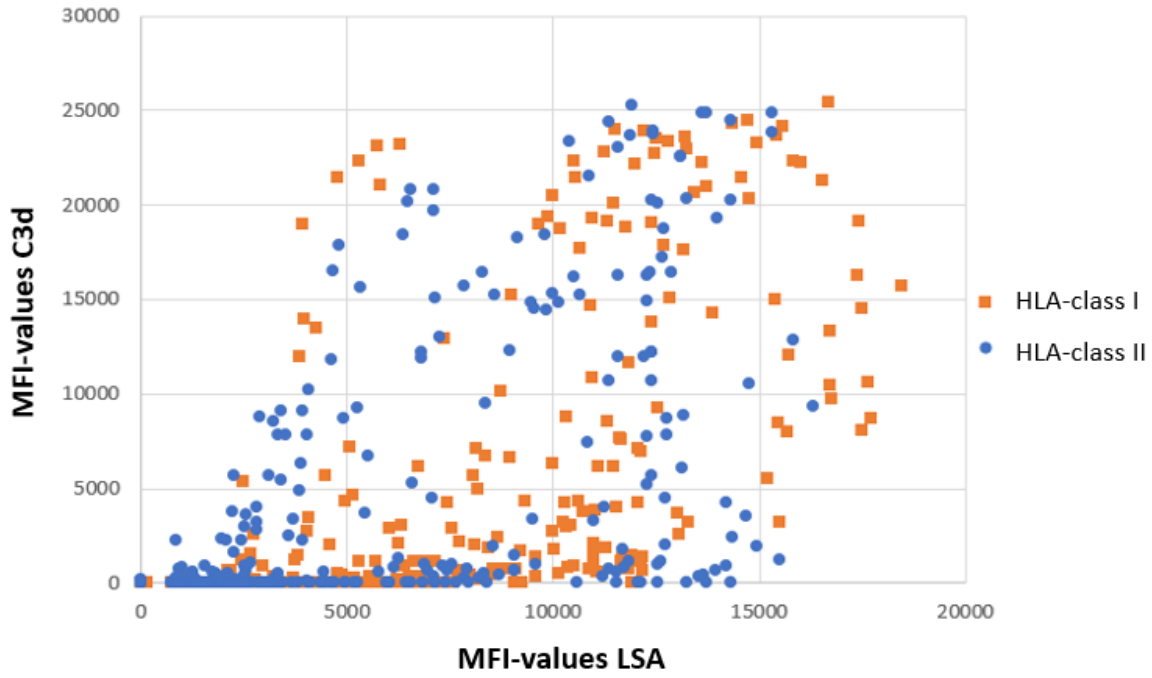

Figure 3 Plot of the MFI-values of all HLA-antibody specificities (LSA) against the corresponding MFI-values of C3d-detection (C3d) for the whole cohort of 74 patients, separated according to HLA-class I-(orange squares) and HLA-class II-(blue circles) specific antibodies. The calculated coefficients of correlation were 0.69 for HLA-class I and 0.63 for HLA-class II-specific signals, thus demonstrating a high correlation for the antibodies directed against the antigens of both HLA-classes.

As expected, the paired values of both HLA-classes were characterized by high mean variations. Both class I- and class II-specific antibodies, however, showed a linear correlation, with the Pearson coefficients calculated to be 0.69 for HLA-class I and 0.63 for HLA-class II-specific antibodies.

Further experiments were performed to substantiate the correlation between the strength of the MFI-values of the antibody specificity and C3d-positivity, representing a dimension of

complement-fixing capacity. For this reason, the sera of three patients were subjected to linear dilution steps. Table 2 shows representative results for patient ID8.

Table 2 Reduction of Luminex-LSA-specific MFI-values and the resulting loss of C3d-positivity as a consequence of increasing serum dilutions (patient ID 8).

HLA-class I Spec.	MFI-LSA	C3d	MFI-LSA	C3d	MFI-LSA	C3d	MFI-LSA	C3d	MFI-LSA	C3d
	undiluted		1:2		1:4		1:8		1:16	
A*01:01	12242	++	11804	++	11473	++	6366	+	5896	+
A*23:01	12170	+	11149	+	8206	+	3007	Ø	2678	Ø
A*24:02	12263	+	11084	+	10004	+	3909	+	3008	Ø
A*26:01	10318	+	6307	Ø	4256	Ø	873	Ø	Ø	Ø
A*29:01	2582	Ø	1763	Ø	907	Ø	Ø	Ø	Ø	Ø
A*29:02	6616	+	2719	Ø	2059	Ø	Ø	Ø	Ø	Ø
A*36:01	13337	++	12398	+	9194	+	2137	Ø	1993	Ø
A*43:01	12062	+	8313	Ø	4630	Ø	947	Ø	Ø	Ø
A*80:01	15514	++	17582	++	15594	++	9189	+	7209	+
B*15:12	11295	++	9342	+	6465	+	1545	Ø	1012	Ø
B*44:02	4128	Ø	2342	Ø	942	Ø	Ø	Ø	Ø	Ø
B*44:03	4500	Ø	2561	Ø	1559	Ø	Ø	Ø	Ø	Ø
B*45:01	5035	Ø	3839	Ø	1845	Ø	Ø	Ø	Ø	Ø
B*82:02	5147	Ø	3502	Ø	2007	Ø	Ø	Ø	Ø	Ø

C3d, classification of C3d-specific MFI-values; ++, C3d-positive with MFI-values ≥10,000; +, C3d-positive with MFI-values <10,000; Ø, C3d-negative; black lettering, C3d-positive and corresponding allele-specific MFI-values; red lettering, C3d-negative and corresponding allele-specific MFI-values.

Eight out of 14 specificities characterized by complement-fixing antibodies were reduced to only two specificities (anti-A*01:01 and anti-A*80:01) over the serum's serial dilution steps between 1:2 and 1:16. Moreover, strong complement-activating antibodies with C3d-specific signals higher than 10,000 (++ in Table 2) could be diluted to C3d-negative results after three steps (Ø in Table 2). This clearly demonstrates the correlation between the titer of the antibodies as indicated by their MFI-values and their capability of activating the complement cascade.

Figure 4 depicts this correlation more clearly, showing antigen-specific MFI values plotted against corresponding C3d-specific MFI values, again exemplified by patient ID8, in which the patient's serum was diluted over serial steps from undiluted to 1:16.

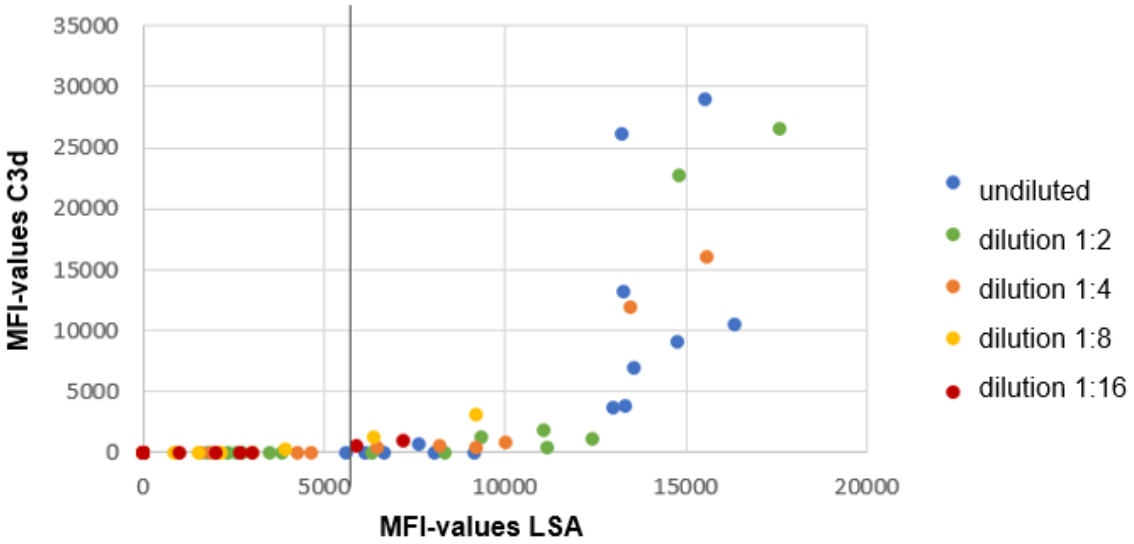

Figure 4 Plots of the MFI-values of HLA-class I antibody specificities (LSA) against the corresponding values of C3d detection (C3d) for patient ID8 using undiluted serum (blue) and four successive serial dilution steps (1:2/green, 1:4/orange, 1:8/yellow, and 1:16/red). The loss of C3d-positivity as a consequence of serum dilution is shown to lead to LSA-specific threshold MFI values for C3d-positivity of between 6,000 and 7,000 (black line).

In good accordance with the data in Figure 2b and Table 2, it is evident that any complement-fixing capability is ultimately lacking as a consequence of dilutions steps that led to antigen-specific MFI values lower than about 6,000. This clear threshold value is indicated by the black vertical line in Figure 4. Sera of two additional patients (ID 13 and ID 24) were diluted and the resulting antigen-specific and C3d-specific MFI values were plotted in the same way (data not shown). The resulting threshold values for C3d-positivity were both between 6,000 and 7,000, thus leading to very similar antigen-specific threshold MFI-values below which no complement-fixing capacity of the antibodies was detectable.

Interestingly, and in contrast to expectations, several HLA-class I specificities, which were identifiable by cell tray analyses (CDC-only-specificities) in eight patients (ID 1, 9, 16, 19, 31, 35, 45, 47), were not definable as complement-activating using the supplementing C3d-assay (Table 3).

Table 3 Antibody specificities identifiable by CDC-based cell-tray analysis, but not by the Luminex-C3d assay due to corresponding MFI-values of LSA-definable specificities below 6,500.

Pat. ID	Spec. CDC	Spec. LSA/MFI-values	Spec. C3d
1	B35	B35/4,100	Ø
9	A69(28)	A69(28)/4,200	Ø
	A68(28)	A68(28)/4,400	Ø
16	B52	B52/5,400	Ø
19	B65	B65/4,800	Ø
	Cw7	Cw7/7,600	Cw7
31	B18	B18/5,600	Ø
35	A23	A23/3,900	Ø
45	B27	B27/5,600	Ø
	B56	B56/4,700	Ø
47	A1	A1/6,400	Ø
	A29(19)	A29(19)/7,900	A29(19)

Spec. CDC, Specificities definable using CDC-based cell-tray analysis; Spec. LSA/MFI-values, Luminex-based single-antigen definable specificities and corresponding MFI-values; Ø, not definable as complement-fixing using the C3d-assay; Spec. C3d, specificities in black lettering, definable as complement-fixing using the C3d-assay.

All of the identified specificities appear valid, as they were all clearly definable using the Luminex single-antigen analysis with antigen-specific MFI-values between 3,900 and 6,400. All of these values, however, are below the threshold value of C3d-positivity delimitable to MFI-values of about 6,500 (Figure 4). In spite of many more antibody specificities definable as complement-activating using the highly resolving C3d-assay, the conclusion must be drawn that this assay lacks sensitivity, which apparently does not even attain the sensitivity of the CDC-based cell-tray analysis depicting antibody specificities with corresponding MFI-values as low as approximately 4,000. This lack of sensitivity independently limits the benefit of the C3d assay, as CDC-based cell-tray analysis for antibody specification is already considered insufficiently sensitive and shows no antibodies with MFI values below 3,000 to 4,000.

Analogous dilution experiments were subsequently performed exemplarily for three patients exclusively depicting HLA-class II-specific antibodies (Pat. ID 67, 72, 75). As expected, due to the HLA-class II-specific coefficient of correlation, which had formerly been calculated as 0.63 (Figure 3), the results of the dilution-dependent correlations of LSA-based antigen-specific MFI values and their resulting positivities in the C3d assay proved to be quite similar. Representative data are shown for patient ID 75 whose serum was investigated using four consecutive dilution steps between 1:2 and 1:16 (Figure 5).

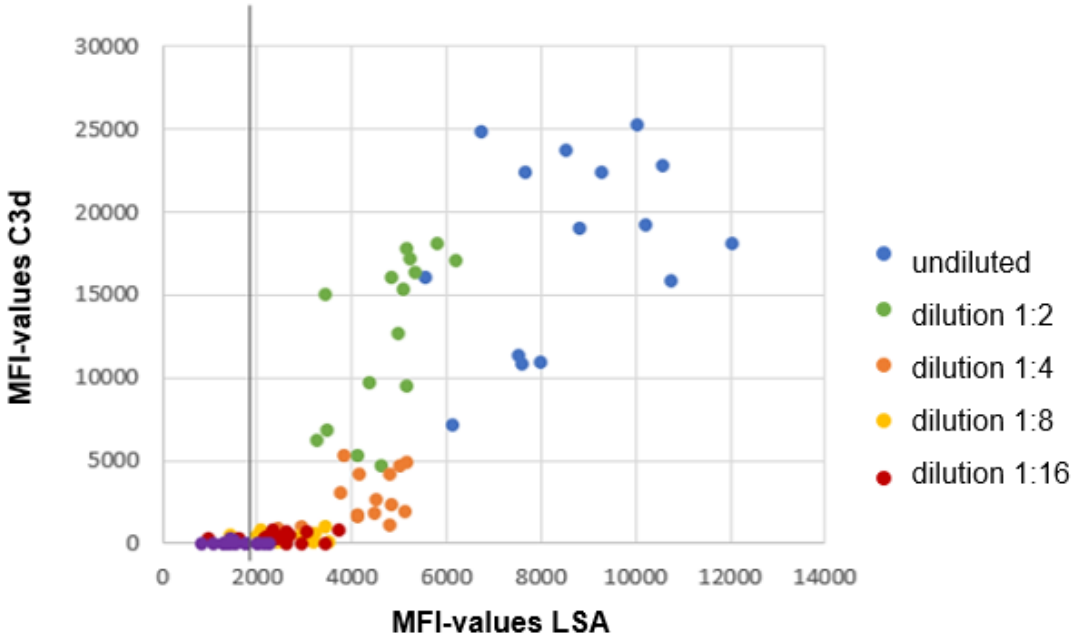

Figure 5 Plots of the MFI-values of HLA-class II antibody specificities (LSA) against the corresponding values of C3d-detection (C3d) of patient ID75 using undiluted serum (blue) and four serial dilution steps (1:2/green, 1:4/orange, 1:8/yellow and 1:16/red). The loss of C3d-positivity as a consequence of serum dilutions is exhibited, leading to LSA-specific threshold MFI-values of C3d-positivity between 1,500 and 2,000 (black line).

Using undiluted serum of patient 75, the Luminex single-antigen specificity assay identified 22 single specificities, of which 17 (77%) were definable as C3d-positive (Figure 5, blue spots). In contrast, after the final 1:16-dilution step, only 11 specificities were identifiable, of which only two (18%) were definable as C3d-positive (Figure 5, red spots). Thus, as also shown for antibodies specific for HLA-class II antigens, the correlation between antibody strength/titer (approximately depicted by MFI-values) and the corresponding capacity to fix complement (depicted by the detectability of C3d) was clearly demonstrable.

However, one striking difference between antibodies directed against one class or the other of HLA antigens should be noted. The antigen-specific threshold value distinguishing C3d-positive from C3d-negative antibodies being in the range of 1,500 to 2,000 was much lower for HLA-class II-specific antibodies. This contrasts with HLA-class I-specific antibodies, which had a substantially higher threshold value, and was the case for all three sera (Pat. ID 67, 72, 75) investigated in this regard (data not shown). Thus, the HLA-class II-specific threshold value came considerably closer to the lower cut-off for raw MFI values (750), which was set to define antibody specificities as either positive or negative. The situation described for eight sera with HLA-class I specificities, which are definable as complement-fixing based on the cell-tray analyses rather than on the Luminex-based C3d assay due to its insufficient sensitivity (Table 3), does not appear to apply to HLA-class II-specific antibodies. Unfortunately, the chronic lymphocytic leukemia (CLL)-based cell trays bearing HLA-class II antigens are no longer commercially available and were thus unavailable for comparative analyses.

Although the reason for the different sensitivity between HLA-class I- and HLA-class II-specific C3d assays could not be resolved, both classes showed threshold values below which all antibodies

without exception lost their complement-activating capacity. Anti-HLA antibodies became increasingly cytotoxic above these thresholds, reaching complete cytotoxicity that paralleled the higher MFI values. Correlation coefficients of 0.69 (HLA-class I) and 0.63 (HLA-class II) thus indicate that the MFI values of the single beads are most probably the main reason for them being classified as cytotoxic. Thus, it should be noted that the C3d assay does not provide independent results, which may be of value in the future for the risk assessment of anti-HLA antibodies.

4. Discussion

Solid phase-based antibody specification systems have considerably improved the quality of antibody diagnoses, with the consequence of improving both pre-transplant and post-transplant prognosis. This has been achieved by better identification of humoral anti-HLA immune responses as the main cause of late-graft loss [32, 33]. The initial investigations by Patel and Terasaki [1], who introduced the CDC-based crossmatch more than fifty years ago, clearly showed that preformed complement-fixing DSA represents a pivotal predictor of hyperacute rejections. Due to limitations in the CDC procedure, particularly regarding sensitivity and specificity, various alternative crossmatch techniques have been developed and implemented during the last decades [8-10, 34-37]. However, the same principle of method is used by the CDC-based cell-tray analysis in order to define antibody specificities, showing the same limitations that generate invalid results. Apart from one publication highlighting the benefit of this assay, but with not entirely plausible arguments [38], the cell-tray analysis has proved unsuitable due to clearly defined methodological drawbacks. These limitations often prevent adequate diagnostic conclusions. The breakthrough was provided by the high-resolution Luminex-based single-antigen assays for antibody specification, which allowed valid virtual cross-matching, but unfortunately did not allow discrimination between complement-fixing and non-complement-fixing antibodies. In those assays, all the IgG isotypes of anti-HLA antibodies are identified by use of pan-IgG-specific polyclonal secondary antibodies. However, it seemed clear that the identification of complement-fixing anti-HLA antibodies may be a more suitable marker to exclude the major cause of graft failure and consequently improve allograft survival [39]. Accordingly, it seemed reasonable to implement a system that combines the specificity and resolution of the Luminex single-antigen assay with the capability to discriminate between highly deleterious complement-fixing antibodies and other antibodies without this activity, which have widely been regarded as minor risk factors for allograft loss.

In our study, we integrated cell-tray analyses of each serum sample, with Luminex-based antibody specification at the corresponding single-donor (SD-) level in order to provide data emphasizing the overall limitations of the CDC-based cell-tray analysis. Although the SD-level of resolution did not correspond to the highest possible single-antigen (LSA-) level of the C3d-assay, other drawbacks of the CDC-assay were clearly demonstrable. As expected, HLA-class II antibodies were not specifiable through the use of PBL-assembled cell-trays in the absence of CLL cell-trays. Due to underlying immune-complex diseases exhibited by some patients, their corresponding CDC-based PRA-values of 100%, 92% and 87%, proved to be over-represented in comparison to the corresponding Luminex single-donor specifications. These failed to identify more than one specificity and delivered PRA values of at most 12%. Furthermore, although the number of antibody specificities deducible from the Luminex SD-assay was higher, due to overlapping specificities, the number of validly identifiable specificities decreased in parallel to the increase in both cell-tray and

Luminex SD analyses. In view of these key drawbacks of the cell-tray analysis, it is unclear why this technique is still used as a diagnostic procedure, which is required to be performed at least annually for all patients on kidney waiting lists supervised by Euro-transplant.

Given the limitations discussed above, complement-activation assays, combined with single-antigen resolution, seem to be a suitable means of better assessing anti-HLA antibodies. It is for this reason that this assessment of the 74 patients as listed above (Table 1) was initiated. Although our findings for both anti-HLA class I and class II antibodies were unexpected at the outset of the study, they are, in hindsight, plausible. Without exception, antibodies were not definable as complement-fixing below MFI-threshold values of 6,000-7,000 for anti-HLA-class I and below values of 1,500-2,000 for anti-HLA-class II antibodies. Although MFI values are not completely representative of their corresponding antibody titers, for reasons discussed elsewhere [40], it can be concluded that the titers of the respective antibodies are the determining factor for being classifiable as complement-activating. Several similar investigations support this conclusion using the C1q-assay from the manufacturer One Lambda, which became commercially available more than ten years ago [41-43]. While one of those initial publications did not find a correlation between the MFI values of single-antigen assays and a positive result in the C1q-assay [41], nevertheless, the striking majority of those initial and subsequent studies clearly substantiated this correlation, which detracted from the potential benefit of using this assay as a supplementary method [44-47].

The second assay developed to identify complement-fixing antibodies, labeled the C3d assay, was launched about three years later by the manufacturer Lifecodes and is commercially available (distributor Werfen/Immucor). Technically, it represented considerable progress in comparison with the earlier C1q assay. The C1q assay uses recombinant C1q, which must be added to bind complement-fixing anti-HLA antibodies and be detected by a fluorophore-conjugated C1q-specific monoclonal antibody. In contrast, the C3d assay detects the complement cleavage product C3d, attached to the Luminex beads, as a consequence of an essential induction of the complement system by the antigen-bound anti-HLA antibodies. For this reason, human serum must additionally be added as a complement source. Thus, the capacity of HLA-specific antibodies to activate the complement cascade is monitored instead of the mere binding of the initial component of the classical pathway of complement activation (Figure 1). In addition, this principle of function is characterized by increased sensitivity than the C1q system.

Our initial aim was to develop a procedure that completely replaces the outdated CDC-based cell-tray analysis and to establish a system to reliably distinguish highly deleterious complement-fixing antibodies from residual antibodies of minor relevance. This goal, however, was not reached in this study. Indeed, several studies have been published, which attributed the capability of identifying patients at increased or high risk of allograft loss to the C3d assay prior to, or at the time of, antibody-mediated rejection [48-52]. All, however, failed to investigate a possible correlation between MFI-values and C3d-mediated signals, although studies adopting this approach have been published [53-55]. Our investigations align with those previous studies, reaching the same conclusion; however, we were unaware of them before starting our investigation.

It is noteworthy that sub-isotype (subclass) IgG1 is dominant among the four IgG-subclasses of IgG1 (78%), IgG2 (49%), IgG3 (36%) and IgG4 (20%). IgG1 and IgG3 are known as strong activators of the complement system. IgG2 is known to activate complement only sparsely, and IgG4 lacks complement-activating capability. Historically pre-transplant DSA were divisible into percentages of 28% for IgG1/IgG3, only 5% for IgG2/IgG4 and 62% for mixed, i.e., both complement-fixing and non-

binding subclasses [56]. Together, 90% of all patients were characterized by DSA composed of strong or a mixture of strong and weak/no complement-activating IgG subclasses with similar clinical outcomes. Although IgG4 and IgG2 subclasses are well known to block the complement activation induced by other subclasses, it is rather improbable that IgG2 and IgG4 dominate the HLA-directed immune response in a manner that completely blocks complement activation. This open issue remains to be clarified in future experiments, especially to determine the *in vivo* situation with respect to the influence of HLA-directed IgG subclasses on complement system activation and to assess the impact of these antibodies on allograft rejection.

5. Conclusion

The vast majority of complement-fixing/cytotoxic antibodies, as detected in the C3d assay, are deducible from their high MFI values in the Luminex-based standard single-antigen assay. This is indicated by Pearson correlation coefficients of 0.69 (HLA-class I) and 0.63 (HLA-class II) between MFI values of antibody specificity and C3d positivity. Furthermore, dilution experiments led to a reduction of C3d-positivity, even to its complete loss. It is noteworthy that our investigations depict individual assay- and cohort-specific threshold values of semi-quantitative assays, characterized by high inter-assay variations of their underlying MFI values. Thus, these values should not be regarded as universally valid biological limits and should not be used to directly assess transplant outcomes by predicting antibody-mediated rejection episodes or graft loss. Nevertheless, our investigations clearly demonstrate that the C3d assay does not provide independent results, which may be of additional value to risk assessment of anti-HLA antibodies. Because of the additional workload, high costs, and lack of apparent benefit, we do not recommend implementing this assay in routine laboratory practice.

Author Contributions

GeSc: conceived the study and developed the research design, supervised the project, wrote the manuscript. AD: performed the experiments and analyzed the data, validated the results and developed the visualisations. GaSa: supervised the project, proofread the manuscript. MR: provided support for statistical analyses.

Competing Interests

The authors have declared that no competing interests exist.

AI-Assisted Technologies Statement

This work was created and written without the use of AI-tools.

References

1. Patel R, Terasaki PI. Significance of the positive crossmatch test in kidney transplantation. N Engl J Med. 1969; 280: 735-739.

2. Ahern AT, Artruc SB, Della Pelle P, Cosimi AB, Russel PS, Colvin RB, et al. Hyperacute rejection of HLA-AB-identical renal allografts associated with B lymphocyte and endothelial reactive antibodies. Transplantation. 1982; 33: 103-106.
3. Chapman JR, Taylor C, Ting A, Morris PJ. Hyperacute rejection of a renal allograft in the presence of anti-HLA Cw5 antibody. Transplantation. 1986; 42: 91-93.
4. Feucht HE, Felber E, Gokel MJ, Hillebrand G, Nattermann U, Brockmeyer C, et al. Vascular deposition of complement-split products in kidney allografts with cell-mediated rejection. Clin Exp Immunol. 1991; 86: 464-470.
5. Feucht HE, Schneeberger H, Hillebrand G, Burkhardt K, Weiss M, Riethmüller G, et al. Capillary deposition of C4d complement fragment and early renal allograft loss. Kidney Int. 1993; 43: 1333-1338.
6. Regele H, Böhmig GA, Habicht A, Gollowitzer D, Schillinger M, Rockenschaub S, et al. Capillary deposition of complement split product C4d in renal allografts is associated with basement membrane injury in peritubular and glomerular capillaries: A contribution of humoral immunity to chronic allograft rejection. J Am Soc Nephrol. 2002; 13: 2371-2380.
7. Feucht HE, Mihatsch MJ. Diagnostic value of C4d in renal biopsies. Curr Opin Nephrol Hypertens. 2005; 14: 592-598.
8. Schlaf G, Pollok-Kopp B, Manzke T, Schurat O, Altermann W. Novel solid phase-based ELISA assays contribute to an improved detection of anti-HLA antibodies and to an increased reliability of pre- and post-transplant crossmatching. NDT Plus. 2010; 3: 527-538.
9. Schlaf G, Pollok-Kopp B, Altermann W. Sensitive solid-phase detection of donor-specific antibodies as an aid highly relevant to improving allograft outcomes. Mol Diagn Ther. 2014; 18: 185-201.
10. Schlaf G, Rothhoff A, Altermann WW. Systemic lupus erythematosus leading to terminal renal failure and excluding patients from kidney allocation due to inadequate CDC-based cross-matching: Is there a diagnostic way out? J Clin Cell Immunol. 2014; 5: 198-206.
11. Rebibou JM, Chabod J, Bittencourt MC, Thevénin C, Chalopin JM, Hervé P, et al. Flow-PRA® evaluation for antibody screening in patients awaiting kidney transplantation. Transpl Immunol. 2000; 8: 125-128.
12. Gebel HM, Bray RA, Ruth JA, Zibari GB, McDonald JC, Kahan BD, et al. Flow PRA to detect clinically relevant HLA antibodies. Transplant Proc. 2001; 33: 477.
13. Kao KJ, Scornik JC, Small SJ. Enzyme-linked immunoassay for anti-HLA antibodies-an alternative to panel studies by lymphocytotoxicity. Transplantation. 1993; 55: 192-196.
14. Worthington JE, Thomas AA, Dyer PA, Martin S. Detection of HLA-specific antibodies by PRA-STAT and their association with transplant outcome. Transplantation. 1998; 65: 121-125.
15. Papassavas AC, Iniotaki-Theodoraki A, Stathakopoulou A, Ioannou S, Kostakis A, Stavropoulos-Giokas C. Evaluation of HLA-class I alloantibodies using PRA-STAT and complement-dependent cytotoxicity techniques. Transplant Proc. 1998; 30: 724-726.
16. Zachary A, 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: 228-235.
17. Colombo MB, Haworth SE, Poli F, Nocco A, Puglisi G, Innocente A, et al. Luminex technology for anti-HLA antibody screening: Evaluation of performance and of impact on laboratory routine. Cytometry B Clin Cytom. 2007; 72: 465-471.

18. Tait BD, Hudson F, Cantwell L, Brewin G, Holdsworth R, Bennett G, et al. Luminex technology for HLA antibody detection in organ transplantation. Nephrology. 2009; 14: 247-254.
19. Launhardt K, Lefevre V, Souplet V, Prantl L, Marget M, Hovoricova B, et al. Concordance with established tests and reproducibility of results obtained with a new single antigen chip array for HLA antibody detection (HISTO SPOT® HLA AB). J Immunol Methods. 2021; 491: 112971.
20. Morales-Buenrostro LE, Terasaki PI, Marino-Vázquez LA, Lee JH, El-Awar N, Alberú J. "Natural" human leukocyte antigen antibodies found in nonalloimmunized healthy males. Transplantation. 2008; 86: 1111-1115.
21. Cai J, Terasaki PI, Anderson N, Lachmann N, Schönemann C. Intact HLA not beta2m-free heavy chain-specific HLA class I antibodies are predictive of graft failure. Transplantation. 2009; 88: 226-230.
22. Pereira S, Perkins S, Lee JH, Shumway W, LeFor W, Lopez-Cepero M, et al. Donor-specific antibody against denatured HLA-A1: Clinically nonsignificant? Hum Immunol. 2011; 72: 492-498.
23. Poli F, Benazzi E, Innocente A, Nocco A, Cagni N, Gianatti A, et al. Heart transplantation with donor-specific antibodies directed toward denatured HLA-A*02:01: A case report. Hum Immunol. 2011; 72: 1045-1048.
24. Süsal C, Ovens J, Mahmoud K, Döhler B, Scherer S, Ruhenstroth A, et al. No association of kidney graft loss with human leukocyte antigen antibodies detected exclusively by sensitive Luminex single-antigen testing: A collaborative transplant study report. Transplantation. 2011; 91: 883-887.
25. Otten HG, Verhaar MC, Borst HP, van Eck M, van Ginkel WG, Hené RJ, et al. The significance of pretransplant donor-specific antibodies reactive with intact or denatured human leucocyte antigen in kidney transplantation. Clin Exp Immunol. 2013; 173: 536-543.
26. Ravindranath MH, Jucaud V, Ferrone S. Monitoring native HLA-I trimer specific antibodies in Luminex multiplex single antigen bead assay: Evaluation of beadsets from different manufacturers. J Immunol Methods. 2017; 450: 73-80.
27. Gloor JM, Winters JL, Cornell LD, Fix LA, DeGoey SR, Knauer RM, et al. Baseline donor-specific antibody levels and outcomes in positive crossmatch kidney transplantation. Am J Transplant. 2010; 10: 582-589.
28. Zachary A, Reinsmoen NL. Quantifying HLA-specific antibodies in patients undergoing desensitization. Curr Opin Organ Transplant. 2011; 16: 410-415.
29. Anani WQ, Zeevi A, Lunz JG. EDTA treatment of serum unmasks complement-mediated prozone inhibition in human leukocyte antigen antibody testing. Am J Clin Pathol. 2016; 146: 346-352.
30. Carey BS, Boswijk K, Mabrok M, Rowe PA, Connor A, Saif I, et al. A reliable method for avoiding false negative results with Luminex single antigen beads; evidence of the prozone effect. Transpl Immunol. 2016; 37: 23-27.
31. Bertrand D, Farce F, Laurent C, Hamelin F, François A, Guerrot D, et al. Comparison of two Luminex single-antigen bead flow cytometry assays for detection of donor-specific antibodies after renal transplantation. Transplantation. 2019; 103: 597-603.
32. Einecke G, Sis B, Reeve J, Mengel M, Campbell PM, Hidalgo LG, et al. Antibody-mediated microcirculation injury is the major cause of late kidney transplant failure. Am J Transplant. 2009; 9: 2520-2531.

33. Stegall MD, Gloor JM. Deciphering antibody-mediated rejection: New insights into mechanisms and treatment. Curr Opin Organ Transplant. 2010; 15: 8-10.
34. Schlaf G, Mauz-Körholz C, Ott U, Leike S, Altermann W. General insufficiency of the classical CDC-based crossmatch to detect donor-specific anti-HLA antibodies leading to invalid results under recipients' medical treatment or underlying diseases. Histol Histopathol. 2012; 27: 31-38.
35. Schlaf G, Apel S, Wahle A, Altermann WW. Solid phase-based cross-matching as solution for kidney allograft recipients pretreated with therapeutic antibodies. Biomed Res Int. 2015; 2015: 587158.
36. Bau D, Sawers G, Wahle A, Altermann W, Schlaf G. Solid phase-based cross-matching using the Luminex platform for solid organ allo-grafting: Rather regression than progress in comparison to the ELISA-based precursor procedure. J Clin Cell Immunol. 2019; 10: 1000576.
37. Schlaf G, Bau D, Horstmann N, Sawers G, Altermann W. Solid phase-based cross-matching for solid organ transplantation: Currently out-of-stock but urgently required for improved allograft outcome. Histol Histopathol. 2020; 35: 937-948.
38. Doxiadis II, Roelen D, Claas FH. Mature wines are better: CDC as the leading method to define highly sensitized patients. Curr Opin Organ Transplant. 2010; 15: 716-719.
39. Bouquegneau A, Loheac C, Aubert O, Bouatou Y, Viglietti D, Empana JP, et al. Complement-activating donor-specific anti-HLA antibodies and solid organ transplant survival: A systematic review and meta-analysis. PLoS Med. 2018; 15: e1002572.
40. Tait BD, Süsal C, Gebel HM, Nickerson PW, Zachary AA, Claas FHJ, et al. Consensus guidelines on the testing and clinical management issues associated with HLA and non-HLA antibodies in transplantation. Transplantation. 2013; 95: 19-47.
41. Llorente S, Boix F, Eguia J, Lopez M, Bosch A, Martinez H, et al. C1q-fixing human leukocyte antigen assay in immunized renal patients: Correlation between Luminex SAB-C1q and SAB-IgG. Transplant Proc. 2012; 44: 2535-2537.
42. Crespo M, Torio A, Mas V, Redondo D, Perez-Saez MJ, Mir M, et al. Clinical relevance of pretransplant anti-HLA donor-specific antibodies: Does C1q-fixation matter? Transpl Immunol. 2013; 29: 28-33.
43. Zeevi A, Lunz J, Feingold B, Shullo M, Bermudez C, Teuteberg J, et al. Persistent strong anti-HLA antibody at high titer is complement binding and associated with increased risk of antibody-mediated rejection in heart transplant recipients. J Heart Lung Transplant. 2013; 32: 98-105.
44. Taylor CJ, Kosmoliaptsis V, Martin J, Knighton G, Mallon D, Bradley JA, et al. Technical limitations of the C1q single-antigen bead assay to detect complement binding HLA-specific antibodies. Transplantation. 2017; 6: 1206-1214.
45. Gebel HM, Bray RA. A diagnostic 'C' saw: The ups and downs of C1q testing. Curr Opin Organ Transplant. 2019; 24: 402-410.
46. Baranwal AK, Goswami S, Agarwal SK, Kaur G, Mehra NK. Impact of C1q fixing donor-specific antibodies on renal transplant outcome. Scand J Immunol. 2021; 94: e13048.
47. Heinemann FM, Lindemann M, Kelez D, Witzke O, Kribben A, Baba HA, et al. Cumulative mean fluorescent intensities of HLA specific antibodies predict antibody mediated rejections after kidney transplantation. HLA. 2022; 100: 553-562.
48. Sicard A, Ducreux S, Rabeyrin M, Couzi L, McGregor B, Badet L, et al. Detection of C3d-binding donor-specific anti-HLA antibodies at diagnosis of humoral rejection predicts renal graft loss. J Am Soc Nephrol. 2015; 26: 457-467.

49. Comoli P, Cioni M, Tagliamacco A, Quartuccio G, Innocente A, Fontana I, et al. Acquisition of C3d-binding activity by *de novo* donor-specific HLA antibodies correlates with graft loss in nonsensitized pediatric kidney recipients. Am J Transplant. 2016; 16: 2106-2116.
50. Zhang Q, Hickey M, Drogalis-Kim D, Zheng Y, Gjertson D, Cadeiras M, et al. Understanding the correlation between DSA, complement activation, and antibody-mediated rejection in heart transplant recipients. Transplantation. 2018; 102: e413-e438.
51. Kim JJ, Shaw O, Martin C, Michaelides G, Balasubramaniam R, Sebire NJ, et al. Clinical risk stratification of paediatric renal transplant recipients using C1q and C3d fixing of the donor-specific antibodies. Pediatr Nephrol. 2018; 33: 167-174.
52. Babu A, Khovanova N, Shaw O, Griffin S, Briggs D, Krishnan NS, et al. C3d-positive donor-specific antibodies have a role in pretransplant risk stratification of cross-match-positive HLA-incompatible renal transplantation: United Kingdom multicentre study. Transpl Int. 2020; 33: 1128-1139.
53. Claisse G, Absi L, Cognasse F, Alamartine E, Mariat C, Maillard N. Relationship between mean fluorescence intensity and C1q/C3d-fixing capacities of anti-HLA antibodies. Hum Immunol. 2017; 78: 336-341.
54. Gonzales MM, Mitema DG, Smith BH, Schinstock CA, Stegall MD, Wakefield LL, et al. Comparison between total IgG, C1q, and C3d single antigen bead assays in detecting class I complement-fixing anti-HLA antibodies. Transplant Proc. 2017; 49: 2031-2035.
55. Ko SY, Lee W, Jung CW, Cho Y. C3d assay in correlation with single antigen bead assay for human leukocyte antigen antibodies. Transplant Proc. 2018; 50: 2354-2358.
56. Hönger G, Hopfer H, Arnold ML, Spriewald BM, Schaub S, Amico P. Pretransplant IgG subclasses of donor-specific human leukocyte antigen antibodies and development of antibody-mediated rejection. Transplantation. 2011; 92: 41-47.