Non-HLA Antibodies May Accelerate Immune Responses After Intestinal and Multivisceral Transplantation

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Background. Non-HLA alloantibodies and autoantibodies are involved in allograft rejection in kidney and heart transplantation. Their role in intestinal transplantation has not yet been described. We examined the development of antiangiotensin II type I receptor antibodies (anti-AT_1R) and antiendothelin type A receptor antibodies associated with the clinical course and histopathological findings of intestinal transplantation recipients. **Methods.** Thirty-seven patients underwent intestinal or multivisceral transplantation. Non-HLA antibodies (non-HLAabs) were screened in 29 transplant recipients. Antibody-levels greater than 12 U/L were considered positive and were evaluated retrospectively regarding rejection episodes. **Results.** Twenty patients developed anti-AT_1R and/or antiendothelin type A receptor antibodies (non-HLAabs group), 9 did not (control group). The non-HLAabs group had a higher rate of allograft rejection than controls (80% vs 55%), especially a higher rate of antibody-mediated rejections (55% vs 11%, $P < 0.01$) with detection of donor-specific anti-HLAabs. All rejection episodes in the non-HLAabs group appeared around the time of positive non-HLAabs detection. Five patients had acute cellular rejections at the time of non-HLAabs development, 4 had viral infections. **Conclusions.** Our data suggest that antibody-mediated mechanisms targeting antigens beyond HLA may trigger and accelerate immune responses. Given the possibility of pharmacologic targeting of non-HLA receptors, future studies will focus on the explanation of mechanisms how non-HLAabs may enhance rejection and affect long-term allograft survival.

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Intestinal immunogenicity is distinguished by a constant colonization with microorganisms, large numbers of resident leukocytes and especially the strong expression of histocompatibility antigens.[1] The continuous improvement of immunosuppression and a better understanding of the mechanisms of allograft rejection have increased short-term survival after intestinal transplantation (ITX), yet chronic allograft injury reduces long-term survival and is still not completely understood.[2] The recognition of dynamic interactions between B and T cells, together with more sensitive HLA donor-specific HLA antibody (DSA) flow cytometric and solid-phase tests directed the focus of attention towards antibody-mediated rejection (AMR). This form of rejection has recently been associated with a poorer outcome, especially in liver-free grafts[3,4] and was thought to be responsible for long-term graft attrition after ITX.[2,4] Multiple therapeutic strategies like targeting T and B cells,[5] removal of antibodies,[6] and immunomodulation of antibody action[7] have been adopted mainly from the field of kidney transplantation (KTX).[8] The complement-split product C4d is a cornerstone in the diagnostic proof of DSA-mediated vascular graft injury in renal transplantation,[9] but has been proven inconclusive in ITX. Yet, even in KTX 40% to 50% of severe vascular rejections are C4d negative[10] and together with the occurrence of allograft rejections in HLA-identical sibling transplants, these findings suggest the involvement of non-HLA and/or noncomplement-fixing antibodies.[11]

Abu-Elmagd et al[4,12] have addressed the same issue in a recently published study on alloantibody assessment and outcome

prediction after intestinal and multivisceral transplantation (MVTX). They state that ongoing rejection in the absence of circulating antibodies could signal coexistence of non-HLAabs or nondonor-specific HLA antibodies (NDSA), which may indicate HLA antibody absorption to the graft.

Because these mechanisms beyond ABO blood group and major histocompatibility complex (MHC) class I chain-related gene A and B antigens have long been recognized in other solid organ transplantations like kidney,[13-15] heart,[16,17] and lung[18,19] transplantation, the diagnostic and therapeutic strategies used here may be translated to the field of ITX. Numerous non–HLA-fixing, complement-fixing, and noncomplement-fixing antibodies have been described to be responsible for various allograft injuries, indicating the complex mechanisms of their action in different organ transplants. Of the variety of antibodies targeting minor histocompatibility antigens, vascular receptors, adhesion molecules, and intermediate filaments, we have investigated the impact of antibodies directed against the angiotensin type 1 receptor (anti-AT_1R) and endothelin type A receptor (anti-ET_AR). Naturally, the impact of non-HLAabs on intestinal graft injury is not yet sufficiently investigated. However, their simultaneous occurrence during rejection episodes, viral infections, or NDSA development as described in this study may suggest an additional involvement of immune responses in rejection processes that has not yet been described.

MATERIALS AND METHODS

Patients who had undergone isolated intestinal (ITX), modified multivisceral transplantation (mMVTX: stomach, duodenum, intestine, pancreas) or typical multivisceral transplantation (MVTX: stomach, duodenum, intestine, pancreas, liver) in the transplant center in Berlin, Germany, between 2000 and 2015 were included in this study.

The following data were collected:

(1) Patient and graft characteristics

These included patient demographics, underlying disease, age, graft type, immunosuppression, time of allograft rejection, number/grade of rejections, time/type of infection, level/time of occurrence of HLAabs/non-HLAabs, type/duration of antirejection treatment and outcome (Table 1).

(2) Induction therapy

In the early years of the ITX program, Daclizumab (Zenapax, Hoffmann-La Roche, Basel, Switzerland; 20 mg intravenously [iv]) and 1 dose of antihuman T-lymphocyte immunoglobulin Fresenius (ATG-FreseniusS; Fresenius-Biotech, Munich, Germany; 8 mg/kg body weight [BW]) were used as induction therapy, which was then changed to Alemtuzumab (Campath, Genzyme, Cambridge, MA; 30 mg iv on postoperative day 1 + 4). Finally, the protocol was modified to thymoglobulin (Thymoglobulin; Genzyme; 7.5 mg/kg BW total dose) and 1 dose of infliximab (Remicade; Centocor Inc., Essex Pharma GmbH; 5 mg/kg BW). Infliximab was used to mitigate ischemia/reperfusion injury and to deplete effector memory $CD8^+$ T cells.[20,21]

(3) Baseline and maintenance immunosuppression

The initial immunosuppression comprised tacrolimus (Prograf Astellas, Japan; initial trough-levels 15-20 ng/mL, tapered to 10-15 ng/mL) and steroids (40 mg/d, tapered off by postoperative day 80). Maintenance immunosuppression included tacrolimus (trough-levels 5-6 ng/mL) and mycophenolate mofetil (Cellcept; Hoffmann-LaRoche, Switzerland; 500 mg or 1000 mg every 12 hours) or sirolimus (Rapamune; Wyeth Ayerst Pharmaceuticals, USA; trough-levels 2-3 ng/mL) depending on proteinuria, wound healing, diarrhea, myelotoxicity.

(4) Determination of anti-AT_1R and anti-ET_AR concentrations in serum

Within the group of non-HLAabs, we screened exclusively for antibodies against the AT_1R and ET_AR. Screening for AT_1R and ET_AR antibodies has been introduced into routine in 2004, thus no data exist on non-HLAabs of the first ITX recipients in our center. Pretransplant non-HLAab monitoring was performed once upon listing for transplant and every 3 months thereafter.

Posttransplant non-HLAabs and HLAabs monitoring was always performed on the same day: once a week and whenever necessary for diagnosis, until discharge from hospital. Outpatients were screened for alloantibodies every 6 months. A serum level of 12 U/mL for both antibody subgroups was defined as clinically relevant cutoff.

A solid-phase sandwich ELISA kit (CellTrend GmbH, Luckenwalde, Germany) was used to measure AT_1R as well as ET_AR antibody levels in patients' serum according to a recently validated protocol.[22] The interassay and intra-assay CVs are 11.5% and 6.9% for the AT_1R and 8.3% and 6.3% for the ET_AR-ELISA kit. The lower limit of detection was 1 U/mL. Serum samples were thawed and centrifuged for 10 min at 1800 rpm before analysis. All samples were subjected to identical freeze-thaw cycles. The test was performed according to the product insert of the manufacturer. The OD450 measurement was conducted using a Multiskan EX Microplate Photometer (ThermoScientific, Rockford, IL). Data analysis was performed using AT_1R/ET_AR analysis software provided by the manufacturer.

(5) Determination of HLAabs

Before transplantation, patients were screened for HLAabs every 3 months by solid-phase assays and once annually by the complement-dependent cytotoxicity (CDC) test. Thus, we defined cytotoxic antibodies by the CDC and used the solid phase assay to identify those antibodies as well as additional cytotoxic and noncytotoxic antibodies. The following 2 different solid-phase assays were performed between 2000 and 2015: (1) ELISA-based Lambda Antigen Trays (LAT) (One Lambda, Canoga Park, CA, USA) until 2006, and (2) Luminex bead-based LABScreen HLAab-detection assays (One Lambda) introduced in 2007. A stepwise approach was used for the detection and identification of HLAabs by solid-phase assays both pre and posttransplant. Initially, we performed screening assays (LAT Mixed or LABScreen Mixed) and in case of a positive result, the HLAabs specificity was subsequently determined by specification-assays (LAT PRA or LABScreen Single Antigen). For this study, all ELISA-based HLAabs tests have retrospectively been reanalyzed by

LABScreen assays. All tests have been performed according to the manufacturer's instructions.

Unacceptable antigens for the 2 patients (nos 13 and 20) with preformed HLAabs before transplantation were defined by LABScreen Single Antigen and CDC. In detail, all specificities as detected by CDC and additional IgG antibodies exceeding 1000 normalized mean fluorescence intensity (MFI) units in the single-antigen bead (SAB) assay have been defined as unacceptable antigens for transplantation. ABO-identical grafts were used exclusively, and transplantation was performed only after a negative pretransplant CDC-based and virtual crossmatch under consideration of the defined unacceptable antigens.

During the posttransplant period, no fixed MFI-based cutoff value was used and DSA MFI units were carefully monitored under consideration of previous serum samples. DSA were determined for the HLA loci A, B, C, DRB1, DRB3/4/5, and DQB1 based on donor and recipient molecular typing. Recipients were typed by sequence-specific oligonucleotide assays. Donor typing, usually performed by sequence-specific primer assays in the donor center, was communicated by Eurotransplant. Patients were considered to have developed de novo DSA when the MFI significantly increased in comparison to previous samples (≥ + 100%) and to a level that was ≥3-fold above the respective negative control, which was revealed to be indicative for HLA-specific antibodies in the SAB assay.[23] De novo DSA were confirmed by testing a subsequent serum sample and the coincidental detection of cross-reactive HLAabs. The relatively low cutoff definition has been proven appropriate for the posttransplant analysis of ITX- and mMVTX-recipients as we aimed at the earliest possible detection of DSA formation and patients exhibited typically relatively low level DSA at this stage. To account for day-to-day variability of SAB test results, we normalized MFI values of DSA with the respective positive control bead. Data were expressed as %MFI.

(6) Diagnosis of Rejection

Rejection was identified by clinical symptoms and confirmed via graft biopsies which were assessed according to established histological rejection criteria.[24] In addition, intestinal graft biopsies were performed as per protocol or as clinically indicated via endoscopy. C4d staining was regularly performed. In the event of AMR, the published potential histopathological signs of humoral rejection were applied.[22,25]

(7) Antirejection Treatment

Steroid therapy was used for mild acute cellular rejection (ACR) (1000 mg methylprednisolone) for 5 consecutive days. For moderate, severe, and steroid-resistant ACR we applied thymoglobulin (1-1.5 mg/kg BW for 5 days to achieve lymphocyte counts less than 500 cells/nL).

Antibody-mediated rejection was treated with plasmapheresis (5 cycles every other day) and alternating IVIGs (10 g/d iv) until the histological and clinical resolution of rejection and the disappearance of DSA were achieved. Rituximab was included in the treatment (MAB THERA, Hoffmann-La Roche, Switzerland; 375 mg/m^2 body surface iv) in case of DSA persistence (despite repeated plasmapheresis/IVIG) and evidence of ongoing rejection. Bortezomib was added as rescue therapy for treatment-refractory AMR (Velcade; Janssen-Cilag, Germany; 1.6 mg/kg BW) on days 1, 4, 8, and 11.[26]

(8) Data Analysis

Data were collected prospectively and obtained by retrospective review of medical records to assess clinical variables and histopathological results from graft biopsies. Continuous data were analyzed by Student *t* test. Ordinal data were assessed by Fisher exact test, where appropriate, or descriptively due to limited patient numbers. The results are provided as the mean ± standard error of the mean.

RESULTS

Thirty-seven patients (13 female, 24 male; 38.2 ± 9.8 years) underwent ITX (n = 21), mMVTX (n = 2) or MVTX (n = 14) as of 2000. AB0 blood group identical grafts were used exclusively and transplantation was performed only after receiving a negative pretransplant CDC-based and virtual crossmatch. Unacceptable antigens for transplantation were defined based on the detected cytotoxic and noncytotoxic HLAabs. Non-HLAabs screening was available for 29 patients, 8 patients were excluded from the study, because they were transplanted prior to routine non-HLA screening.

Appearance of Non-HLAabs After ITX or MVTX May Be Associated With Different Immune Activations

In a retrospective analysis, 20 of 29 patients (69%) were found to have non-HLAabs, whereas 9 patients (31%) were not (Figure 1).

We subdivided these 29 transplant recipients into 2 groups:

(a) Non-HLAabs group (Table 1: Patients who developed non-HLAabs): n = 20

ITX = 9; MVTX = 9; mMVTX = 2

(b) Control group (Table 2: Patients who did not develop non-HLAabs): n = 9

ITX = 5; MVTX = 4; mMVTX = 0

The detected non-HLAabs were directed against AT_1 receptors or ET_A receptors or both.

In 12 cases (41%), these were de novo non-HLAabs. Eight patients showed non-HLAabs before transplantation, which disappeared beyond detection but reemerged at different times after transplantation (Table 1). We observed that the appearance or reappearance of non-HLAabs after transplantation was always synchronized with events of immune activation, such as allograft rejection or infection. Whether there is a definite causative link between these observations or whether they are mere epiphenomena needs further investigation.

Non-HLAabs May Trigger Allograft Rejection

Once detected, the levels of non-HLAabs did not show any clear dynamic of increase or decrease. Instead, the levels varied, so that we calculated means for each patient and each patient group. Mean levels of non-HLAabs during ACR episodes were 8.6 ± 13.0 for anti-AT_1R and 15.9 ± 4.2 for anti-ET_AR. For AMR episodes the mean values were 14.7 ± 2.1 for anti-AT_1R and 15.7 ± 5.8 for anti-ET_AR. With regard to the association of allograft rejection and

TABLE 1.

Characteristics of patients with Non-HLA antibody development

Patient characteristics			Immunosuppression		Non-HLAabs				
No	Age at TX (years) + graft type	Underlying disease	Induction therapy	Maintenance IS at the time of non-HLA detection	Pre-TX (mean, U/L) Anti-AT_1R	Pre-TX (mean, U/L) Anti-ET_AR	Post-TX (mean, U/L) Anti-AT_1R	Post-TX (mean, U/L) Anti-ET_AR	Time of development post-TX
1	27 ITX (2004)	Congenital malrotation	ATG-F, Dac	Tac Sirolimus MMF	4.77 ± 1.3	5.21 ± 0.7	5.3 ± 1.0	12.4 ± 0.4	5 y
2	36 MVTX + KTX (2005)	Crohn disease	Alemtuzumab	Tac MMF	<2.5	<2.5	13.9 ± 0.8	19.2 ± 1.9	5 y
3	39 ITX (2005)	Mesenterial infarction	Alemtuzumab	Tac, steroids	7.39 ± 0.9	<2.5	12.9 ± 0.7	6.9 ± 1.2	4 wk
4	31 (2007)	CIPO	Infliximab TG	Tac, Steroids	7.22 ± 2.2	12.9 ± 0.3	8.77 ± 1.0	13.1 ± 1.0	4 wk
5	24 MVTX + KTX (2007)	Congenital volvulus	Infliximab, TG	Tac MMF	7.39 ± 1.3	6.78 ± 2.1	5.46 ± 0.9	13.0 ± 1.0	6 mo
6	36 MVTX (2007)	Road accident	Infliximab, TG	Tac, Steroids	13.9 ± 1.6	33.9 ± 3.5	17.6 ± 5.6	21.0 ± 10.9	2 wk
7	23 ITX (2008)	CIPO	Infliximab, TG	Tac, Steroids	<2.5	14.3 ± 1.6	<2.5	13.5 ± 0.8	4 wk
8	21 mMVTX (2008)	CIPO	Infliximab, TG	Tac, Steroids	18.3 ± 2.1	36.8 ± 5.8	15.9 ± 1.2	17.6 ± 2.2	4 wk
9	42 MVTX (2008)	Gardner syndrome	Infliximab, TG	Tac, Steroids Tac, MMF	<2.5	5.43 ± 0.8	13.1 ± 0.4	17.6 ± 0.7	4 wk
10	38 ITX (2008)	Mesenterial infarction	Infliximab, TG	Tac, Steroids	<2.5	<2.5	12.5 ± 0.7	15.8 ± 2.0	4 wk
11	45 ITX (2009)	Adhesive ileus	Infliximab, TG	Tac, Sterodis	7.51 ± 1.0	8.09 ± 0.8	13.4 ± 1.4	17.1 ± 4.8	4 wk
12	38 mMVTX (2010)	CIPO	Infliximab, TG	Tac, Steroids	15.1 ± 1.2	22.9 ± 3.5	12.5 ± 0.7	14.7 ± 2.8	4 wk
13	49 MVTX (2010)	Mesenterial fibromatosis	Infliximab, TG	Tac, Steroids	7.14 ± 1.1	5.12 ± 2.3	14.6 ± 0.8	16.2 ± 3.6	4 wk
14	37 MVTX (2010)	Crohn disease	Infliximab, TG	Tac, Steroids Tac, MMF	<2.5	13.5 ± 0.5	14.6 ± 2.5	18.0 ± 8.5	4 wk
15	48 MVTX (2011)	Adhesive ileus	Infliximab, TG	Tac, Steroids	6.95 ± 2.6	<2.5	14.7 ± 2.5	21.7 ± 3.7	4 wk
16	51 MVTX (2012)	Gardner syndrome	Infliximab, TG	Tac Everolimus	5.67 ± 1.3	4.39 ± 2.5	14.2 ± 1.5	12.4 ± 0.5	7 mo
17	56 MVTX (2013)	Gardner syndrome	Infliximab, TG	Tac, Steroids	16,9 ± 2.9	14,8 ± 1.4	29.0 ± 11.0	21.6 ± 5.9	4 wk
18	30 ITX (2013)	CIPO	Infliximab, TG	Tac Everolimus	29 ± 2.4	23 ± 5.1	14.3 ± 1.8	18.6 ± 7.3	4 wk
19	46 ITX (2014)	Adhesive ileus	Infliximab, TG	Tac, everolimus	3.85 ± 0.9	5.91 ± 2.1	18.9 ± 0.9	12.6 ± 2.8	4 wk
20	39 ITX (2014)	Desmoid	Infliximab, TG	Tac Everolimus	<2.5	<2.5	13.9 ± 0.8	17.9 ± 2.5	3 wk

Characteristics of patients who developed non-HLAabs, also displaying the serum levels of anti-AT_1R and anti-ET_AR in association to the time of their development and the simultaneously appearing immune reaction. In addition, the timely correlation between the development of donor-specific HLAabs (DSA) and non-HLAabs is given. The serum levels of non-HLAabs are given as well as the MFI of DSA.
ATG-F, antihuman T-lymphocyte immunoglobulin Fresenius; CIPO, chronic intestinal pseudo-obstruction; Dac, daclizumab; EBV, Ebstein-Barr Virus; IS, immunosuppression; MMF, mycophenolate mofetil; MOF, multiorgan failure; PP, plasmapheresis; PTLD, posttransplant lymphoproliferative disorder; Tac, tacrolimus; TG, thymoglobulin.

the appearance of non-HLAabs, we made 4 different observations:

(a) Patients who developed non-HLAabs had a higher risk of allograft rejection than controls (80% vs 56%; Table 3).
(b) Rejections, which appeared upon positive non-HLAabs sampling, were antibody-mediated in 55%. In fact, a subanalysis showed that the AMR rate was significantly higher in the non-HLAabs group (55% vs 11%), whereas the ACR rate was higher in the control group (44% vs 25%; Table 3). Furthermore, in patients with a higher degree of AMR, non-HLAabs were detected before HLAabs (Table 1).
(c) The mean number of HLA class II antigen mismatches (HLA-DR and HLA-DQ) in patients who developed non-HLAabs was significantly higher than in controls

HLAabs		Immunological event		Therapy	Outcome
HLAabs Pre-TX	Time of develop-ment Post-TX	Allograft rejection around the time of HLA- and non-HLAabs detection	Infections at non-HLAabs detection	Antirejection/antiviral treatment	Patient graft survival
None	n.a.	**ACR I°**	None	Steroids	Survival
None	n.a.	ACR I° of KTX	None	Steroids	Survival
None	5 wk	AMR III° DSA (MFI/%MFI): A2 (6735/45), B35 (2522/17), B51 (3754/25), DR4 (1443/12)	None	Steroids, TG, IVIG, PP, rituximab	Graft loss, MOF, death
None	n.a.	ACR I°	None	Steroids	Survival
None	n.a.	ACR I° of KTX	None	Steroids	Survival
None	4 wk	AMR II° DSA (MFI/%MFI): *B8 (7773/41)*	None	Steroids, TG, IVIG, PP, Rituximab	Survival
None	n.a.	None	Norovirus NDSA	IVIG	Survival
None	5 wk	**AMR III°** DSA (MFI/%MFI): DR15 (1399/9), DR16 (1430/10), DR51 (2180/15)	None	Steroids, TG, IVIG, PP	PTLD, death
None	n.a.	None	Rotavirus NDSA	IVIG	Survival
None	3 wk	AMR I° DSA (MFI/%MFI): *DQ7 (6060/52), DQ8 (3938/34)*	None	Steroids, TG, IVIG, PP, Rituximab	Survival
None	1 wk	AMR I° DSA (MFI/%MFI): A24 (1186/10), DQ7 (4278/41), DQ8 (2457/27), DR53 (4390/42)	None	IVIG, PP, Rituximab, Bortezomib	Survival
None	5 wk	AMR II° DSA (MFI/%MFI): DR4 (5830/30), DR53 (3974/21), DQ8 (7394/39)	None	Steroids, TG, IVIG, PP, Rituximab,	Survival
NDSA class I, DSA (MFI/% MFI): B60 (225/1)	4 wk	AMR II° DSA (MFI/%MFI): B60 (2672/23)	None	Steroids, IVIG, PP, Rituximab,	Survival
None	n.a.	None	EBV NDSA	IVIG	Survival
None	4 wk	AMR II° DSA (MFI/%MFI): B7 (2810/29), DQ7 (3337/27)	None	Steroids, IVIG, PP,	Survival
None	n.a.	None	CMV NDSA	IVIG antiviral treatment	Survival
None	n.a.	ACR I°	None	Steroids,	Survival
None	5 wk	AMR III° DSA (MFI/%MFI): A24 (981/8), DQ7 (1251/12), DQ8 (2138/21)	None	Steroids, IVIG, PP, Rituximab,	Survival
None	3 wk	AMR I° DSA (MFI/%MFI): A24 (3918/34), A32 (5691/50), B57 (8078/71), DQ9 (3584/30)	None	Steroids, IVIG, PP, Rituximab,	Survival
NDSA class I, DSA (MFI/% MFI): DQ6 (424/4)	2 wk	AMR II° DSA (MFI/%MFI): DQ6 (3428/29)	None	Steroids, IVIG, PP, Rituxi-mab, Bortezomib	Survival

(3.2 ± 0.9 vs. 1.6 ± 1.5; $P < 0.0001$; Table 3). We had made similar observations in prior investigations with our cohort, where patients with posttransplant DSA and subsequent AMR showed significantly more class II antigen mismatches (HLA-DR and HLA-DQ) than controls.[23]

(d) Almost all reported rejection episodes were directed against the intestinal graft. In the non-HLAabs group; however, 2 MVTX recipients rejected their kidney graft by ACR at the time of non-HLAabs detection, but not the intestine (Table 1).

Non-HLAabs May be Involved in Viral Infections of the Intestinal Allograft

We noticed that patients who developed non-HLAabs without having an associated rejection (n = 4) had viral

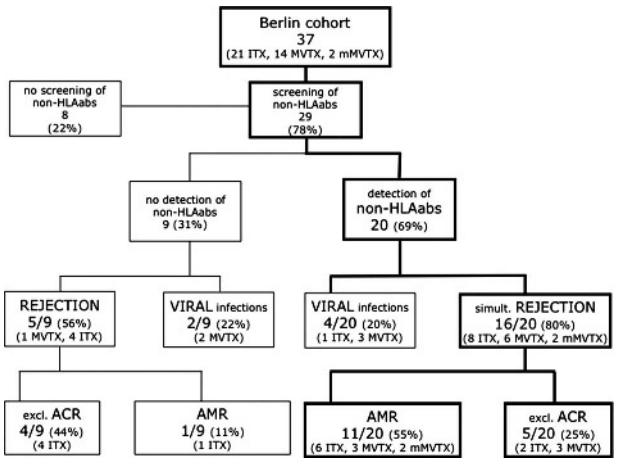

FIGURE 1. The Berlin cohort consists of 37 isolated intestinal, modified or typical multivisceral transplant recipients. Twenty of them developed non-HLAabs simultaneously to immune reactions like antibody-mediated rejections, cellular rejections, or viral infections.

infections. Interestingly, these patients also had de novo HLAabs at the same time, which were NDSA and therefore not directed against the graft (Table 1).

Antibody levels were 10.5 ± 7.0 for anti-AT_1R and 15.4 ± 2.8 for anti-ET_AR. Allograft infections were due to rotavirus, norovirus, cytomegalovirus, and Ebstein-Barr virus and did not correlate with any augmentation of immunosuppression.

In contrast, the infection rate in the control group was not significantly different (20% vs 22%, Table 3), but here infections correlated with antirejection treatment and were possibly due to overimmunosuppression.

DISCUSSION

The clinical relevance of humoral immune responses beyond major histocompatibility antigens in KTX has been confirmed in several studies.[13-15,27]

Recent experimental data as well as the discovery of new antigen targets have warranted more acceptance for non-HLAabs in graft injury and rejection, drawing attention to their impact in other solid organ transplantations like heart,[17,28] lung,[18,29] and lately also composite tissue transplantation.[30] Therefore, it seems likely that similar effects of non-HLAabs may play a role in a highly immunogenic organ like the intestine. Despite this well-known immunogenicity and the constant threat of rejection, humoral immune responses to HLAabs have only recently been recognized[31] and acknowledged to increase the risk of rejection in ITX recipients.[4,22] Non-HLAabs in the setting of ITX have not been previously studied.

To our knowledge, this is the first study that aims to investigate the immunological circumstances in which non-HLAabs develop or reemerge in patients after ITX even in the presence of liver transplant in the context of MVTX.

We are aware of certain shortcomings in our study. Eight patients had to be excluded from the study, because they had not been screened for non-HLAabs. Also, the control group was smaller than the non-HLAabs group, so that in the light of an overall small patient number, significances were hardly reached. We were not able to investigate non-HLAabs binding to the intestinal allograft to prove their direct involvement in the rejection or infection process, so that

TABLE 2.

Control group: Patients after ITX/MVTX, who did not develop any Non-HLA antibodies

No	Age at TX Years	Graft Type + Year of TX	Underlying disease	Induction	Maintenance immunosuppression	Grade of rejection	Antirejection treatment	Other events	Outcome
1	49	ITX (2004)	Gardner syndrome	ATG-F, daclizumab	Tacrolimus, sirolimus	—	—	Pseudomembranous enterocolitis	Death in MOF
2	31	ITX (2005)	Mesenterial infarction	ATG-F, daclizumab	Tacrolimus, sirolimus	AMR II° DSA (MFI): A3(1830) A24(2336) DQ7 (7974)	Steroids, thymoglobulin, rituximab, bortezomib	—	Graft loss, alive
3	28	ITX (2005)	Volvulus	ATG-F, daclizumab	Tacrolimus, sirolimus	ACR I°	Steroids	—	Alive
4	31	ITX (2005)	Adhesive ileus	Alemtuzumab	Tacrolimus, sirolimus	ACR II°	Steroids, OKT 3, infliximab	—	Alive
5	44	ITX (2009)	Mesenterial infarction	Thymoglobulin, infliximab	Tacrolimus, sirolimus	ACR II°	Steroids, thymoglobulin, infliximab	—	Alive
6	29	MVTX + KTX (2011)	Crohn disease	Thymoglobulin, infliximab	Tacrolimus, sirolimus	—	—	—	Alive
7	52	MVTX (2011)	Adhesive ileus	Thymoglobulin, infliximab	Tacrolimus, everolimus	—	—	*Campylobacter jejuni* infection	Alive
8	33	MVTX (2013)	Crohn disease	Thymoglobulin, infliximab	Tacrolimus, everolimus	—	—	Aspergillosis, adenovirus infection	Alive
9	24	MVTX (2014)	CIPO	Thymoglobulin, infliximab	Tacrolimus, everolimus	ACR III°	Steroids thymoglobulin	Candidiasis	Death in MOF

TABLE 3.

Comparison of non-HLA and control group

	Non-HLA group	Control group	*P*
Patient number	20	9	—
Overall rejection rate	16/20 (80%)	5/9 (56%)	n.s.
ACR	5/20 (25%)	4/9 (44%)	n.s.
AMR	11/20 (55%)	1/9 (11%)	0.01
HLA class II mismatches	3.2 ± 0.9	1.6 ± 1.5	0.0001
Intestinal infection rate	4/20 (20%)	2/9 (22%)	n.s.
Mortality rate	2/20 (10%)	2/9 (22%)	n.s.

Comparison of non-HLA and control group, depicting the rejection and mortality rate. A differentiation between ACR and AMR is given as well as the number of HLA class II mismatches.

epiphenomena cannot be excluded. However, the larger patient number in the non-HLAabs group does show that the development of non-HLAabs after ITX and MVTX may be more frequent and more relevant than expected.

We observed that on the one hand, patients who developed non-HLAabs in general had a greater risk of developing a rejection compared to controls and on the other hand, that non-HLAabs only appeared or reappeared in association with immune responses like allograft rejection or infection. The most striking result was that 80% of the non-HLAabs group developed non-HLAabs in timely correlation to histologically proven rejections, and that they had significantly more AMR than controls. In comparison, cellular rejections were more frequent in the control group, who had not developed non-HLAabs. As DSA appeared relatively early at 1 to 5 weeks posttransplant, they may have emerged from plasma cell differentiation of preformed memory B cells or as a result of primary B cell activation. Unfortunately, we cannot discriminate between both sources due to the retrospective nature of the study. However, regardless the source of DSA, both processes may have been influenced by other ongoing inflammatory responses directed against AT_1R and ET_AR.

These findings may reveal an increased humoral immune response which corresponds to recent reports from kidney and heart transplantation: Taniguchi et al[32] observed a significantly higher risk for graft failure after KTX in patients with both, anti-AT_1R and DSA than in patients with DSA alone. They also confirmed that both DSA and anti-AT_1R were independent predictors of poor graft survival, with de novo anti-AT_1R representing the highest risk of graft failure. Furthermore, a correlation between the different patterns of increasing, fluctuating, or decreasing levels of non-HLAabs and a corresponding risk of graft failure has been described in other studies.

Another study on heart transplant recipients found that the combination of DSA and anti-AT_1R had an increased negative impact on freedom from AMR and ACR than either of the antibody alone. These results were seen over a period of 3 years posttransplant.[33] According to our data, there seem to be certain patients who are prone to such accelerated immune responses through DSA and non-HLAabs. It is well known that class II HLA mismatches, especially HLA-DR, increase the risk for humoral immune responses.

We found a significant association between a high number of HLA class II mismatches and the posttransplant development of non-HLAabs, an association which we had already detected in the same cohort regarding the posttransplant DSA development.[23] These findings imply that a pretransplant risk stratification may help to detect and treat such patients at an early stage before immune activation has reached the stage of rejection.

In our study, we were not able to define a specific kinetics pattern of preexisting and de novo non-HLAabs. Recent studies suggest that preexisting non-HLAabs may result in the development of HLA class II DSA, and thereby increase the risk of AMR.[34] However, we could not find a significant correlation in our study due to its retrospective nature and small patient numbers. Interestingly, we observed that patients with a high-grade AMR (II° and III°) had developed non-HLAabs before HLAabs, which may show a triggering effect of non-HLAabs in certain patients resulting in a stronger immune response. Although considering the low levels of DSA, it is possible that the DSA were being adsorbed by donor antigens and were thus not yet detectable. Nevertheless, we carefully suggest that the described appearance of non-HLAabs is associated with rejection episodes, but not necessarily causative. It is also plausible that these functional antibodies via specific receptor interactions detrimentally influence intestinal endothelium and epithelium, making it more prone to HLA antibody attack, even in the absence of complement mediated mechanisms.

Furthermore, we did not find a significant correlation between the development of non-HLAabs and the underlying disease or graft type. There is a potential risk of cross-reactivity between the development of non-HLAabs and factors causing autoimmune diseases. Seven of 20 patients with non-HLAabs had autoimmune diseases (chronic intestinal pseudo-obstruction, n = 5; Crohn n = 2), but not all of them developed a timely associated rejection. However, the patients with chronic intestinal pseudo-obstruction showed preformed non-HLAabs before transplantation and showed rather severe AMR, so that it would be interesting to further investigate possible interactions between the different antibodies.

It is often suggested that multivisceral grafts containing the liver tend to reject less, because the liver has an immunoprotective effect. This trend was also shown in DSA development and AMR.[35] However, although 50% of our patients had undergone MVTX, we could not find any significant differences in the non-HLAabs development of ITX or MVTX recipients. Similarly, in combined liver and KTX, AMR of the kidney occurred despite presence of the liver.[36] Interestingly though, by the time of non-HLA development, 2 of the MVTX recipients showed a rejection of their kidney graft but not of their intestinal graft. Given the high immunogenicity of the intestine, these findings are rather surprising and could be due to the fact that anti-AT_1R and anti-ET_AR target vascular antigens.

Vascular allograft injury in relation to graft dysfunction and graft rejection has largely been examined in heart and KTX, but not in ITX. In fact, further investigation toward vascular graft injury in ITX is often hampered because the mesenterial vasculature is difficult to examine without risking graft perforation, so that it often only becomes evident after resection of the intestinal graft after surgery. Whether intestinal endothelin receptor antibodies play a role in the setting of rejection and inflammation remains to be clarified. Some data suggest that intestinal endothelins are

potent inflammatory mediators. As polyfunctional cytokines, they induce the adhesion of circulating leucocytes to venous endothelium, an initial step in the pathogenesis of a cellular infiltrate in inflammatory bowel disease.[37] Experimental data showed that blocking endothelin receptors entails a reduced adhesion of leucocytes and reduced inflammation in colonic submucosal venules. On the other hand, ET-1 was suggested to be produced by colorectal cancers and ET(A) antagonists are indicated as potential anticancer agents.[38]

Further investigations will clarify whether non-HLAabs directed at intraluminal or mucosal antigens may be relevant to cause similar intestinal graft dysfunction.

Another phenomenon that we observed was the association between the development of non-HLAabs and viral infections. Although not well defined, several possible mechanisms for the association between viral infections and allograft rejection or dysfunction have been suggested. One of the major findings supporting this thesis is that anti-CMV prophylaxis and preemptive therapy were significantly associated with reduction of acute rejection after solid organ transplantation.[39] A proposed mechanism is that CMV enhances graft rejection by its ability to induce MHC antigen expression. In a rat model, CMV upregulated MHC II antigen expression on the surface of heart endothelial cells and MHC II antigens were displayed on most tubular and all endothelial cells during CMV disease.[40,41] In our study, patients with non-HLAabs who had correlating virus infections also showed a new development of anti-HLA antibodies, which were not directed against the donor, implying that a certain MHC II antigen upregulation may have taken place. However, the interactions between the immunological mechanisms of virus infection and the development of non-HLAabs and NDSA did not result in DSA-development and allograft rejection.

CONCLUSIONS

The presented study shows for the first time that humoral immune responses beyond major histocompatibility antigens may play a role in ITX or MVTX. We witnessed timely correlations between the development of non-HLAabs and immune reactions like antibody-mediated or cellular rejections as well as viral infections. Whether these immune responses are initiated or accelerated by the presence of non-HLAabs needs to be clarified. Yet, patients with higher numbers of HLA class II mismatches seem to be more at risk to develop non-HLAabs and DSA, which makes them more susceptible for AMR. Given the possibility of pharmacologic targeting of AT_1R and ET_AR, future studies will focus on the explanation of mechanisms how non-HLAabs may enhance allograft rejection and decrease long-term allograft survival after ITX or MVTX.

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