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ORIGINAL ARTICLE

ABO desensitization affects cellular immunity and infection control after renal transplantation

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The authors declare no conflict of interest.

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Summary

The impact of ABO desensitization on overall immunity, infectious control, and alloreactivity remains unknown. We compared 35 ABO-incompatible kidney transplant recipients (KTRs) to a control of 62 ABO compatible KTRs. Samples were collected before, at +1, +2, +3, +6, and +12 months post-transplantation. CMV-, BKV-specific, and alloreactive T cells were measured using an interferon-γ ELISPOT assay. The extent of immunosuppression was quantified by enumeration of lymphocyte subpopulations and cytokines. No differences were observed for 5-year allograft survival and function between both groups ($P > 0.05$). However, ABO-incompatible KTRs were more likely to develop CMV infection, BKV-associated nephropathy, and severe sepsis ($P = 0.001$). Interestingly, ABO-incompatible KTRs with poor HLA-match showed the highest rates of infections and inferior allograft function ($P < 0.05$). CD3+, CD4+ T-cell counts, interferon-γ and IL-10 levels were lower in ABO-incompatible KTRs early post-transplantation ($P < 0.05$). Likewise, ABO-incompatible KTRs showed impaired BKV- and CMV-specific T-cell immunity ($P < 0.05$). ABO-incompatible KTRs showed lower frequencies of alloreactive T cells ($P < 0.05$). Our data suggest T-cell depletion due to ABO desensitization, which may contribute to the increased risk of T-cell-dependent infections. Elimination of B cells serving as antigen-presenting cells, thereby causing impaired T-cell activation, plays a significant role in both impaired infection control and reduced alloreactive T-cell activation.

Introduction

Desensitization for ABO-incompatible (iABO)-transplantation must pursue two strategies, pretransplant removal of anti-A/B antibodies and inhibition of post-transplant anti-A/B antibody reappearance. In contrast to HLAs, blood group antigens do not require T-cell sensitization to induce antibody production and are poor inducers of specific T-cell responses due to overall lower immunogenicity [1,2]. Therefore, administration of a single-dose of the anti-CD20 antibody rituximab, antigen-specific immunoadsorptions, and intravenous immunoglobulin (IVIG) in addition to triple-drug maintenance immunosuppression led to excellent long-term results after iABO transplantation [3–7].

Despite conflicting data regarding rate and severity of infectious complications in kidney transplant recipients (KTRs) receiving ABO desensitization, a trend toward higher incidences of bacterial, viral, and fungal infections has been reported [8–12]. Reports on treatment of autoimmune diseases using rituximab suggest a direct effect on T-cell immunity in two ways: (i) reduction of T-cell number due to co-expression of low levels of CD20 in a small number of T- and NK cells and (ii) reduction of T-cell activation by down-regulation of the co-stimulatory molecules CD40 and CD80 on B cells [13–15]. In addition, antigen-specific T-cell response has been shown to be dampened in the presence of IVIG due to decreased internalization of immune complexes inside antigen-presenting cells [16]. These expected effects of ABO desensitization on overall

T-cell immunity, however, have not been investigated in the setting of iABO transplantation following induction and maintenance immunosuppression.

Therefore, it was our goal to address following questions: (i) What is the impact of ABO desensitization on cellular immunity? (ii) What is the impact of ABO desensitization on virus-specific immunity? (iii) What is the impact of ABO desensitization on alloreactive T cells?

Patients and methods

Patients

This study was approved by our local ethics review committee in compliance with the Declaration of Helsinki (Ethic Committee Charité University Medicine Berlin, Germany, 126/2001, 07/30/2001). Informed consent was obtained from all patients. We included 35 consecutive adult solitary KTRs who underwent iABO-kidney transplantation between 2005 and 2012. One hundred and fifty-six ABO compatible (cABO) living donor kidney transplantations were performed during this period, and a control of 62 KTRs was built. Here, only KTRs with IL-2R antagonist induction and triple-drug regimen with tacrolimus, mycophenolate mofetil (MMF), and methylprednisolone were included, leaving 80 of 156 cABO-KTRs. KTRs with insufficient screening for BKV and CMV, or lost to follow-up during the first post-transplant year were excluded from analysis, leaving 62 of 156 cABO-KTRs for analysis (Fig. 1a).

We compared outcomes of patient survival, death-censored graft survival, and graft function. Patients were followed until graft loss, death, or their last patient follow-up. Estimated glomerular filtration rate (eGFR) was calculated by the abbreviated MDRD equation: $186 \times (\text{creatinine}/88.4) - 1.154 \times (\text{age}) - 0.203 \times (0.742 \text{ if female})$. Clinical and infectious characteristics are shown in Table 1, Table S1 and S2.

ABO desensitization protocol

KTRs considered for iABO transplantation received a single-dose (375 mg/m^2) of anti-CD20 antibody rituximab 4 weeks before transplantation. Six days before scheduled transplantation, iABO-KTRs underwent antigen-specific immunoadsorption (Glycosorb A/B columns) to remove isoagglutinin antibodies, until antidonor isoagglutinin (anti-A and/or anti-B) IgG titer decreased to a level of 1:8. Postoperative antigen-specific immunoadsorption was performed on days +3, +6, and +9. Immunoadsorption was only continued if there was a rise in isoagglutinin antibody titer or histological evidence of antibody-mediated rejection. IVIG (0.5 ml/kg) was administered 1 day ahead of the scheduled transplantation and at day +9 post-transplantation.

Immunosuppressive therapy

All patients received induction with IL-2R antagonist (basiliximab), which was given before transplantation and +4 days. Maintenance immunosuppression was a triple-drug regimen with tacrolimus, MMF, and methylprednisolone. Target tacrolimus trough levels were 8–10 ng/ml for the first 6 months, 5–7 ng/ml from 7 to 12 months, and 4–6 ng/ml thereafter. All KTRs received an initial MMF dose of 1000 mg twice daily as tolerated. Methylprednisone was tapered to 4 mg daily over 2–4 weeks post-transplantation.

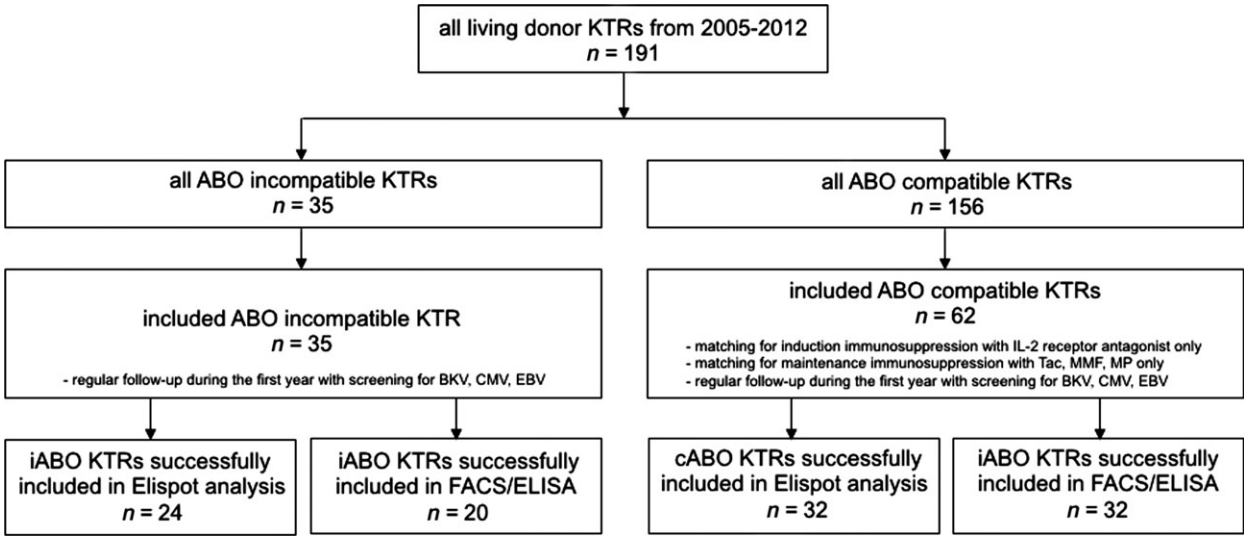

Figure 1 Overview of the study design with enrollment of KTRs in ELISPOT analysis, flow cytometry, and ELISA.

Table 1. Clinical characteristics: ABO compatible (cABO) versus ABO incompatible (iABO).

	ABO compatible (all) (n = 156)	cABO matched (n = 62)	iABO (n = 35)	P value
Age, years*	47 (19–77)	51 (19–73)	52 (18–65)	0.851
Male sex, *n* (%)	106 (68)	46 (74)	23 (77)	0.484
First kidney allograft, *n* (%)	145 (93)	56 (90)	30 (86)	0.519
Time of follow-up, months*	46 (0–107)	37 (1–107)	42 (3–106)	0.560
Causes of ESRD, *n* (%)				
Glomerulonephritis	60 (38)	26 (42)	12 (34)	0.520
Diabetic nephropathy	11 (7)	5 (8)	3 (9)	1
Nephroangiosclerosis	16 (10)	7 (11)	4 (11)	1
Polycystic kidney disease	13 (8)	5 (8)	4 (11)	0.718
Uropathy	7 (4)	4 (6)	3 (9)	0.700
Other or undetermined	49 (31)	15 (24)	9 (26)	1
Diabetes mellitus, *n* (%)	23 (15)	10 (16)	7 (20)	0.782
BMI <18.5 or >30, *n* (%)	20 (13)	9 (15)	6 (17)	0.774
Time on dialysis, months*	10 (0–107)	15 (0–86)	21 (0–116)	0.569
Malignancies, *n* (%)	9 (6)	5 (8)	2 (6)	1
Delayed Graft Function, *n* (%)	9 (6)	4 (6)	5 (14)	0.277
Immunosuppression, *n* (%)				
Tacrolimus	82 (52)	62 (100)	35 (100)	1
Mycophenolate mofetil	156 (100)	62 (100)	35 (100)	1
Steroids	156 (100)	62 (100)	35 (100)	1
Induction therapy, *n* (%)				
IL-2 receptor antagonist	153 (98)	62 (100)	35 (100)	1
Acute rejection 1st year, *n* (%)				
T cell-mediated rejection	39 (25)	16 (26)	7 (20)	0.623
IA/IB	29 (19)	10 (16)	5 (14)	1
IIA/IIB/III	10 (6)	6 (10)	2 (6)	0.707
Antibody-mediated rejection	6 (4)	3 (5)	1 (3)	1
Acute rejection treatment, *n* (%)				
Steroids	29 (19)	10 (16)	7 (20)	0.782
Lymphocyte depletion	8 (5)	6 (10)	2 (6)	0.707
2 HLA-A mismatch, *n* (%)	27 (17)	16 (26)	12 (34)	0.485
2 HLA-B mismatch, *n* (%)	53 (34)	26 (42)	19 (54)	0.291
2 HLA-DR mismatch, *n* (%)	41 (26)	20 (32)	10 (29)	1
Total HLA mismatch, *n* (%)				
4–6 HLA mismatch	61 (39)	31 (50)	24 (69)	0.090
PRA, *n* (%)				
0–10%	154 (99)	62 (100)	35 (100)	1
Donor age, years*	53 (25–78)	52 (25–78)	50 (33–64)	0.323
Donor male sex, *n* (%)	65 (42)	30 (48)	12 (34)	0.484

*Median (range).

Infection monitoring and prophylaxis

Screening for CMV-, EBV-, and BKV load in serum was performed monthly until +6 months, then 3-monthly until +12 months, and yearly thereafter. In addition, screening for CMV- and BKV load was performed at any unexplained rise in serum creatinine and in case of acute rejection.

All patients with a high-risk CMV constellation (D+R−) received a prophylaxis with valganciclovir for 3 months post-transplantation. Oral prophylaxis for pneumocystis jirovecii pneumonia with trimethoprim/sulfamethoxazole was administered 6 months post-transplantation.

Collection of samples for immune monitoring

Fifty-two KTRs of the examined study population were successfully enrolled in our immune monitoring analysis (Fig. 1a). Blood samples for immune monitoring were collected at the following points in time: pretransplantation, +1 week, +1, +2, +3, +6, and +12 months post-transplantation. Citrate blood from donors was collected pretransplantation. PBMC were isolated from 30 to 40 ml of citrate blood using standard Ficoll–Hypaque density gradient technique. Donor PBMC and recipient PBMC were used as stimulator and responder cells, respectively. Sample collection was successful in 20 of iABO-KTRs and 32 of cABO-KTRs.

Fifty-six KTRs of the examined study were successfully enrolled in our ELISPOT analysis. Blood samples for ELISPOT analysis were collected at the following points in time: pretransplantation, +1, +2, and +3 months posttransplantation. Sample collection was successful in 24 iABO-KTRs and 32 cABO-KTRs. BKV-specific immune monitoring was started in 2007, and 15 iABO-KTRs were successfully included.

All KTRs included in immune monitoring (FACS, ELISA) and were the same KTRs included in ELISPOT analysis.

Flow cytometry

Cell phenotype was analyzed by staining with fluorochrome-conjugated monoclonal antibodies for the surface markers CD3, CD4, CD8, CD19 (BD Biosciences, Heidelberg, Germany) to determine lymphocyte subpopulations. Four-color flow cytometry was performed using FACSCalibur and CELLQuest Software (BD Biosciences).

ELISA for IFNγ, IL-2, IL-4, IL-5, IL-10, and TNFα

IFNγ, IL-2, IL-4, IL-5, IL-10, and TNFα serum levels were determined by a sandwich enzyme immunoassay using a human Interferon gamma quantikine, human interleukin 2 quantikine and human tumor necrosis factor alpha quantikine by R&D Systems (Minneapolis, MN, USA).

Design of BKV- and CMV-specific overlapping peptide pools

BKV-strain AS, genotype III, and CMV strain AD169 were used to design BKV- and CMV-specific overlapping peptide pools [17,18]. Overlapping peptide pools were synthesized by JPT (Berlin, Germany) to span the entire sequences of BKV large T-antigen and BKV-VP1, and CMV-pp65 and CMV-IE-1. Overlapping peptide pools were diluted in dimethyl sulfoxide and used at concentrations of 1 μg/ml.

ELISPOT assay for IFNγ detection of BKV- and CMV-specific T cells, and alloreactive T cells

BKV- and CMV-specific cellular immunity were determined by measuring IFNγ upon stimulation of PBMC as described previously [17–19]. For ELISPOT assay, 96-well multiscreen filter plates (MAIPS 4510; Millipore, Schwalbach, Germany) were coated with 100 μl of primary IFNγ monoclonal antibody (mAb) at a concentration of 3 μg/ml (ahu-IFNγ-Endogen M700A) and incubated overnight at 4 °C. A standardized responder T-cell number of 3.0×10^5 PBMC per well were added in duplicate wells with peptides (1 μg/ml), 3.0×10^5 T-cell-depleted donor PBMC, and with Staphylococcus enterotoxin B (SEB; 1 μg/ml; SIGMA, Hamburg, Germany) as positive control and incubated for 24 h at 37 °C. Negative controls were always run in parallel using responder cells plus medium and dimethyl sulfoxide (DMSO). Plates were incubated overnight at 4 °C with 100 μl (1 μl/ml) biotinylated detection IFNG antibody (ahu-IFNG biotin-Endogen M701). After adding streptavidin (1 μg/ml) for 2 h at room temperature, spots were developed by adding 200 μl visualization solution, AEC (3-amino-9-ethylcarbazole, SIGMA) in acetate buffer supplemented with H_2O_2 30% for 3–5 min. Resulting spots were counted using a computer-assisted ELISPOT reader (Immunospot, Cellular Technologies, Ltd., Cleveland, OH, USA). Positive ELISPOT signals were predefined as containing at least 25 spot-forming units per well after subtraction of negative control.

Statistical methods

Statistical tests were performed using SPSS Version 19 (SPSS, Chicago, IL, USA). For comparisons of study groups, two-sided Mann–Whitney *U*-test for nonparametric independent samples was used. For comparisons between paired samples, two-sided Wilcoxon signed-rank test for nonparametric dependent samples was used. Outcomes were measured with Kaplan–Meier models and overall strata comparisons measured by log-rank tests. Univariate and multivariate stepwise logistic regression analyses were performed to assess risk for infection. Clinical and infectious characteristics were compared across groups using Fisher's exact test or chi-square test for categorical variables, and Student's *t*-test for continuous variables. Box plots show median, interquartile range, and 95th percentile. Two-sided $P < 0.05$ were considered statistical significant.

Results

Clinical characteristics

Altogether 35 iABO-kidney transplantations were performed. Median follow-up after transplantation was 42 months (range 3–106 months), during which 5 KTRs died (14.3%) and 2 returned to dialysis (5.7%). 3 iABO-KTRs died from infectious complications (8.6%), 1 iABO-KTR from cardiovascular disease, and 1 KTR of cancer. 1 KTR died of septic shock due to staphylococcal pneumonia at + 39 months, 1 KTR of severe sepsis due to E. coli urinary tract infection at + 31 months, and 1 KTR of Pneumocystis jirovecii pneumonia at + 8 months. Directions of ABO incompatibility were as follows: AB to A in 1, AB to B in 2, A to B in 3, A to O in 14, B to A in 5, and B to O in 10. A control group of 62 cABO-KTRs showed a median follow-up after transplantation of 37 months (range 1–107). Four KTRs died (6.5%) and 5 returned to dialysis (8.1%). Three KTRs died from cardiovascular disease and 1

KTR of cancer. No differences for surgical complications including wound healing, lymphoceles, or bleeding complications were observed ($P > 0.05$). No differences were observed for tacrolimus trough levels and MMF dosing at any time post-transplantation ($P > 0.05$) (Table 2).

No differences for patient survival were observed between iABO- and cABO-KTRs, or any subgroups (Fig. 2a). Death-censored graft survival was comparable for iABO- and cABO-transplantation and any subgroup (Fig. 2b). iABO-KTRs showed a lower eGFR at any time, which did not reach significance (Fig. 2c). Interestingly, iABO-KTRs with 4–6 HLA mismatches showed the worst outcome with a significantly lower eGFR compared to iABO-KTRs with 0–3 HLA mismatches at +60 months ($P = 0.042$).

The highest incidence of infectious complications (defined as CMV viremia, BK viremia, and septic complications) was observed in the early post-transplant period in iABO-KTRs with 60.0% of cases occurring within in the first 2 months compared to only 20.0% of cases in cABO-KTRs (Fig. 2d). Onset of infectious complications was earlier in iABO- compared to cABO-KTRs ($P = 0.038$). iABO-KTRs showed higher incidences of CMV viremia, BKV-associated nephropathy, and severe sepsis (defined as multiorgan dysfunction). No differences were observed for proteinuria or the incidence of antibody-mediated rejection/donor-specific antibodies between iABO-KTRs and cABO-KTRs, or any subgroup ($P > 0.05$). Proteinuria at +12, +36, and +60 months was 172, 174, 183 mg/day for iABO-KTRs, and 168, 148, 156 mg/day for cABO-KTRs, respectively ($P > 0.05$). Five of 35 iABO-KTRs (14.3%) and 9 of 62 cABO-KTRs (14.5%) showed donor-specific antibodies at +60 months after transplantation ($P = 1$).

Risk factors associated with development of infectious complications among all 191 KTRs were analyzed by univariate and multivariate regression analysis. Using univariate analysis, the following five factors were significantly ($P < 0.05$) associated with a higher rate of infectious complications: age, body mass index (BMI), pre-existing or new-onset diabetes, HLA mismatch, and iABO transplantation. Using multivariate analysis factors independently associated with infectious complications within the first 12 months after living donor renal transplantation included iABO transplantation only (hazard ratio [HR] 3.8, 95% confidence interval [CI] 2.8 to 5.6; $P < 0.001$). Age, pre-existing or new-onset diabetes, and HLA mismatch did not reach statistical significance ($P = 0.293$, $P = 0.787$, $P = 0.061$). No differences concerning infectious complications were observed between KTRs with blood group O and KTRs with blood group non-O ($P > 0.05$).

Immunological characteristics

The depleting effect of rituximab treatment in iABO-KTRs was shown by a significant decrease in CD19+ B cells at

Table 2. Infectious complications: ABO compatible (cABO) versus ABO incompatible (iABO).

	cABO matched ($n = 62$)	ABO incompatible ($n = 35$)	*P* value
CMV seropositivity, *n* (%)	39 (63)	21 (60)	0.830
CMV viremia, *n* (%)	13 (21)	16 (46)	0.020*
Peak CMV viremia (copies/mL)*	4.2×10^3 (1.3×10^3–6.5×10^5)	3.2×10^3 (1.0×10^3–5.4×10^5)	
CMV D+R–, *n* (%)	6 (10)	5 (14)	0.519
CMV disease, *n* (%)	4 (6)	6 (17)	0.161
Transplant age at CMV viremia, months*	2 (0–9)	1 (0–11)	0.546
BK viremia, *n* (%)	6 (10)	8 (23)	0.130
Peak BK viremia (copies/mL)*	9.2×10^3 (4.5×10^3–8.7×10^4)	2.4×10^4 (7.1×10^3–8.4×10^6)	–
BK nephropathy, *n* (%)	0 (0)	3 (9)	0.044*
Transplant age at BK viremia, months*	4 (2–10)	3 (1–12)	0.395
EBV viremia, *n* (%)	7 (11)	0 (0)	0.047*
Peak EBV viremia (copies/mL)*	1700 (1.0×10^3–5.5×10^3)	–	–
PTLD, *n* (%)	1 (2)	0 (0)	1
Transplant age at EBV viremia, months*	6 (3–12)	–	–
Septic complications, *n* (%)	3 (5)	5 (14)	0.132
Severe sepsis/septic shock	0 (0)	4 (11)	0.015*
Site of infection, *n* (%)			
Urinary tract infection	2 (3)	2 (6)	0.618
Pneumonia	1 (2)	2 (6)	0.295
Others/unknown	0 (0)	1 (3)	0.361
Transplant age at sepsis, months*	4 (2–8)	3 (0–5)	0.359
Deaths from septic complications, *n* (%)	0 (0)	3 (9)	0.044*
Any infection, *n* (%)	18 (29)	22 (63)	0.001*

*Median (range).

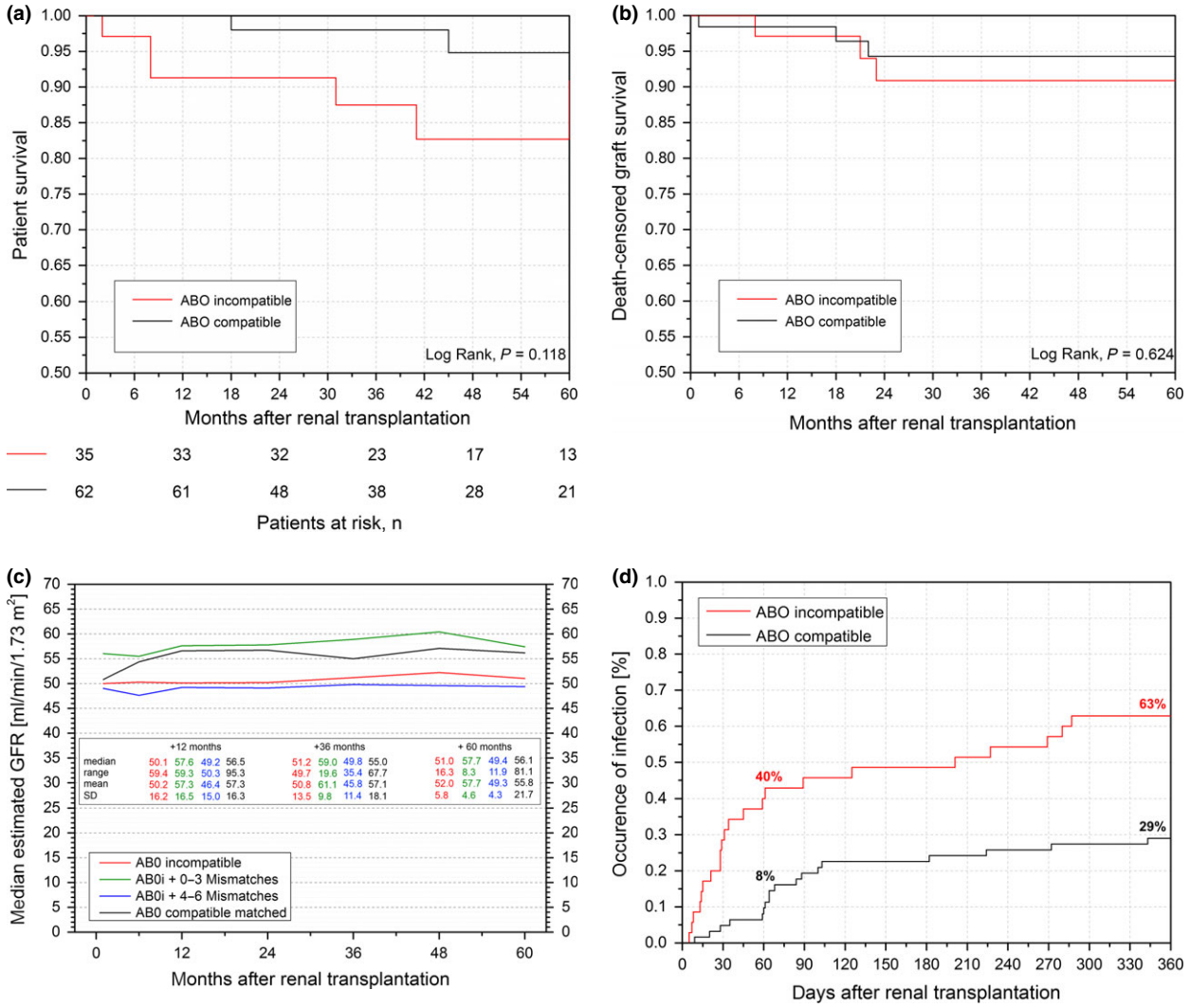

Figure 2 (a) Kaplan–Meier plot of patient survival in ABO-compatible and ABO-incompatible transplantation. Tendency for reduced patient survival in ABO-incompatible transplantation (Log-Rank, $P = 0.118$). (b) Kaplan–Meier plot of death-censored graft survival in ABO-compatible and ABO-incompatible transplantation. Comparable death-censored graft survival in the ABO compatible compared to the ABO-incompatible group (log-rank, $P = 0.624$). (c) Lowest median eGFR in patients with ABO incompatibility + HLA mismatches. No differences are observed between ABO compatible and ABO-incompatible transplantation at any time ($P > 0.05$). (d) Onset of infection (CMV viremia, BK viremia, septic complications) in ABO-compatible and ABO-incompatible transplantation. Earlier onset of infection in ABO incompatible (40% of KTRs) compared to ABO compatible (8% of KTRs) transplantation ($P = 0.028$). Significantly higher rate of infections in ABO incompatible (63% of KTRs) compared to ABO compatible (29% of KTRs) transplantation ($P < 0.001$).

transplantation and during the first post-transplant year ($P < 0.05$). No differences were observed for total CD3+ T-cell counts prior to transplantation between iABO-KTRs and cABO-KTRs, or any subgroup ($P > 0.05$). iABO-KTRs, however, showed significantly lower CD3+ and CD4+ T-cell counts at +60 and +90 days compared to cABO-KTRs ($P < 0.05$; Fig. 3a). iABO-KTRs developed significantly lower IFNγ and IL-10 serum levels compared to cABO-KTRs at +90 days ($P < 0.05$; Fig. 3b,c). CD3+, CD4+, and CD8+ T-cell counts in iABO-KTRs who developed septic complications were significantly lower at the time prior to sepsis compared to cABO-KTRs ($P < 0.05$). No differences were observed between iABO-KTRs developing sepsis and iABO-KTRs not developing sepsis at any time ($P > 0.05$.)

Virus-specific cellular immunity

Analyses of CMV- and BKV-specific immunity were performed during the different steps of iABO desensitization (Fig. 4).

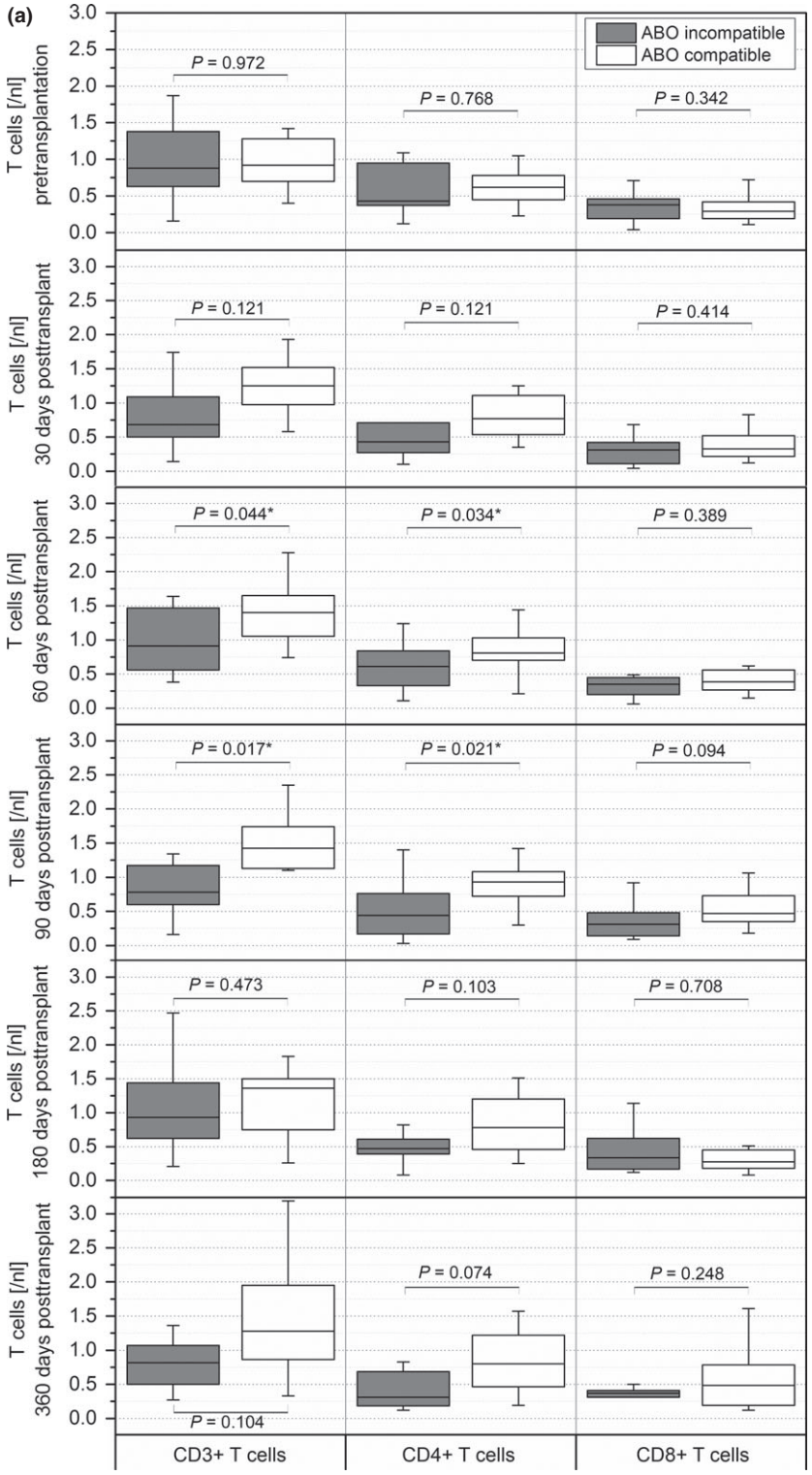

Figure 3 (a) CD3+ Lymphopenia with decreased CD4+ T-cell counts predisposing patients to infectious complications after ABO-incompatible transplantation. Significantly decreased CD3+ and CD4+ T-cell counts characterize patients after ABO desensitization. (b) Decreased IFNγ serum levels in KTRs after ABO-incompatible transplantation. (c) Decreased IL-10 serum levels in KTRs after ABO-incompatible transplantation.

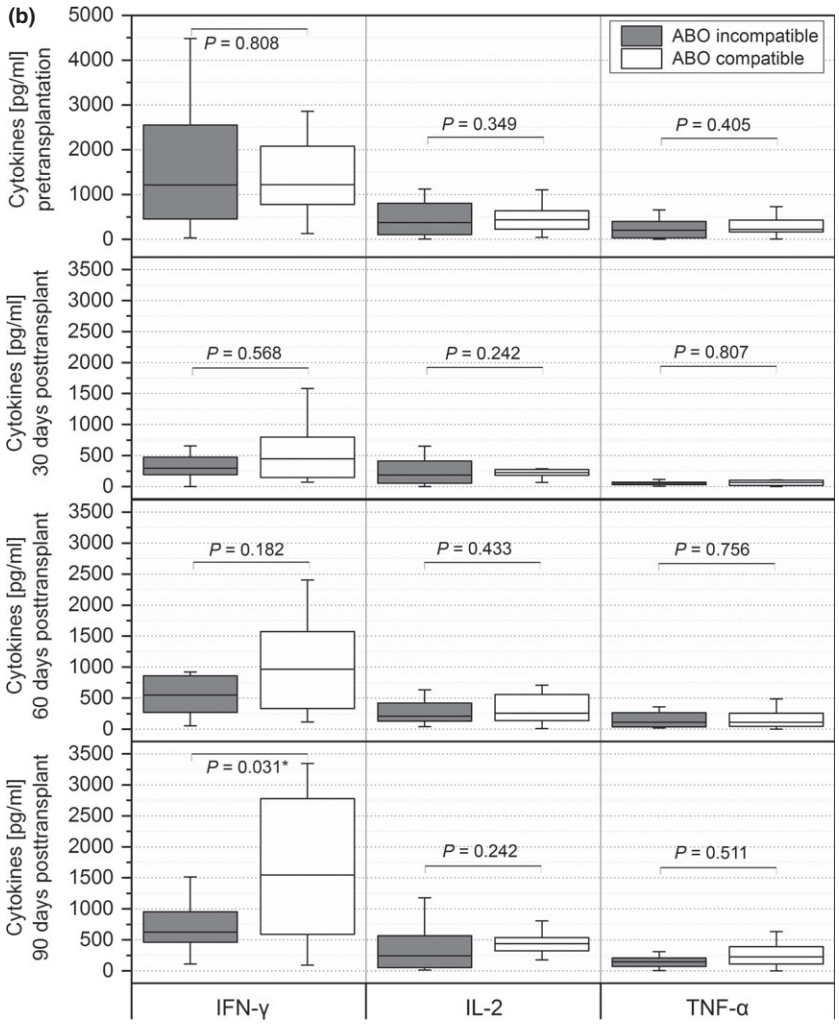

Figure 3 Continued

CMV-specific cellular immunity

Thirteen of 24 iABO-KTRs (54%) showed well-detectable frequencies of CMV-pp65- and/or CMV-IE1-specific T cells prior to transplantation. Whereas no differences were observed for CMV-pp65-specific T cells after rituximab therapy ($P = 0.126$), CMV-IE1-specific T cells were significantly decreased ($P = 0.008$). Interestingly, 8 of 24 iABO-KTRs (33%) showed well-detectable frequencies of CMV-pp65-specific T cells after ABO desensitization, whereas CMV-IE1-specific T cells were not detectable in any KTR after ABO desensitization. While 10 of 24 iABO-KTRs (42%) showed recovery of CMV-pp65-specific T cells after transplantation, only 2 of 24 iABO-KTRs (8%) developed CMV-IE1-specific T cells ($P = 0.034$). Interestingly, 10 of 13 iABO-KTRs (77%) with well-detectable CMV-specific T cells, who lost protective CMV-specific immunity directed CMV-IE1 after ABO desensitization, developed early onset CMV viremia. Three of 5 iABO-KTRs (60%) who were CMV-seronegative and received a kidney from a CMV-seropositive donor developed CMV viremia after discontinuation of CMV prophylaxis at +8, +12, and +13 months. None of these KTRs were able to develop CMV-pp65- or CMV-IE1-specific T cells during our study period.

Due to ABO desensitization significantly less iABO-KTRs showed well-detectable CMV-specific immunity prior to transplantation compared to cABO-KTRs ($P > 0.05$; Fig. 5a). No differences were observed for CMV-specific cellular immunity between iABO-KTRs before rituximab and c-ABO-KTRs before transplantation ($P > 0.05$).

Seventeen of 32 cABO-KTRs (53%) showed well-detectable frequencies of CMV-pp65- and/or CMV-IE1-specific T cells prior to transplantation (Fig. 5a). cABO-KTRs developing CMV viremia showed a significant increase in

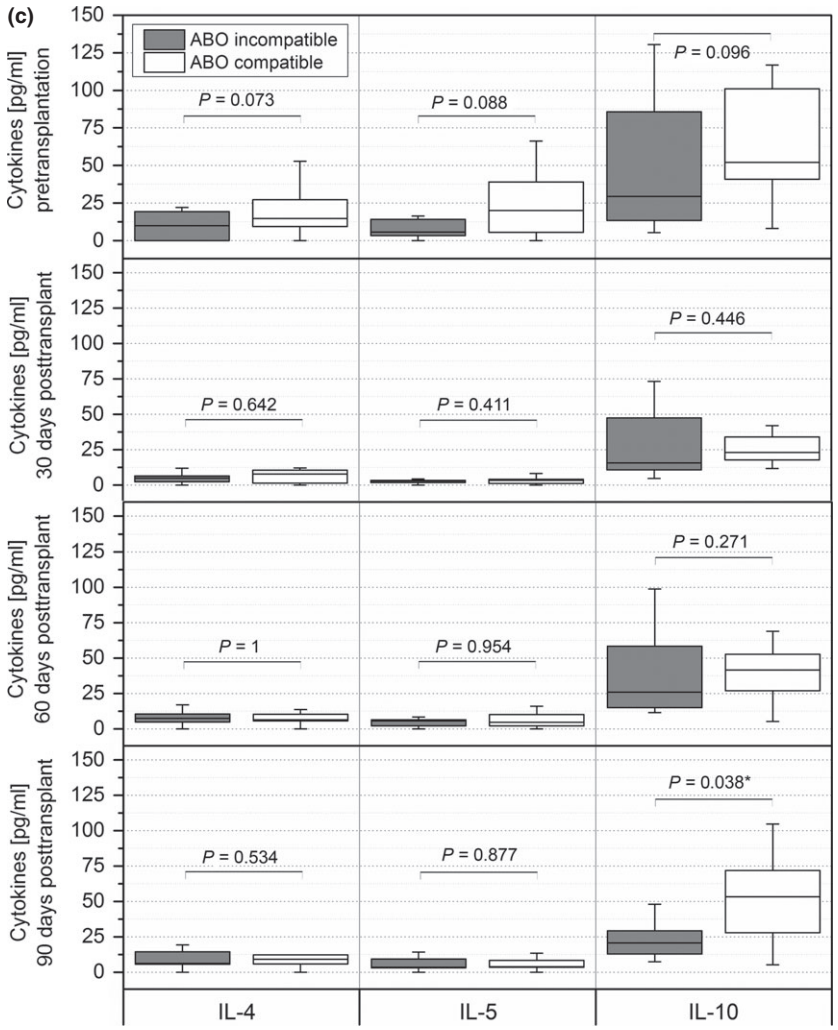

Figure 3 Continued

CMV-specific T cells directed to CMV-pp65 and CMV-IE1 ($P > 0.05$). Five of 15 cABO-KTRs (33%) without CMV-specific immunity directed to CMV-pp65- and/or CMV-IE1 developed early onset CMV viremia. Two of 4 cABO-KTRs (50%) who lost protective CMV-specific immunity directed CMV-IE1 developed early onset CMV viremia. No KTRs (0%) with persistent CMV-specific immunity directed to CMV-IE1 developed CMV viremia in follow-up.

Two of 6 cABO-KTRs (33%) who were CMV-seronegative and received a kidney from a CMV-seropositive donor developed CMV viremia after discontinuation of CMV prophylaxis at +7 and +11 months. In contrast to iABO-KTRs, 3 of these 6 cABO-KTRs (50%) were able to develop well-detectable CMV-pp65- and CMV-IE1-specifc T cells during our study period and did not develop CMV viremia in follow-up.

BKV-specific cellular immunity

Four of 15 iABO-KTRs (27%) showed well-detectable BKV large T-specific and BKV-VP1-specific T cells prior to transplantation. These BKV-specific immune responses decreased in all KTRs and were undetectable at transplantation. Interestingly, 3 of 4 KTRs with pretransplant BKV-specific T cells showed early onset BK viremia. Two of these KTRs were not able to expand a BKV-specific immune response in the early post-transplant period and showed progression to BKV-associated nephropathy. One KTR showed self-limited BK viremia with expansion of BKV-specific T cells at +60 days and 1 KTR without detectable BK viremia showed expansion of BKV-specific T cells at +30 days. Two KTRs with undetectable pretransplant BKV-specific T cells developed self-limited BK viremia.

Five of 25 cABO-KTRs (20%) showed well-detectable frequencies of BKV large T-specific and BKV-VP1-specific

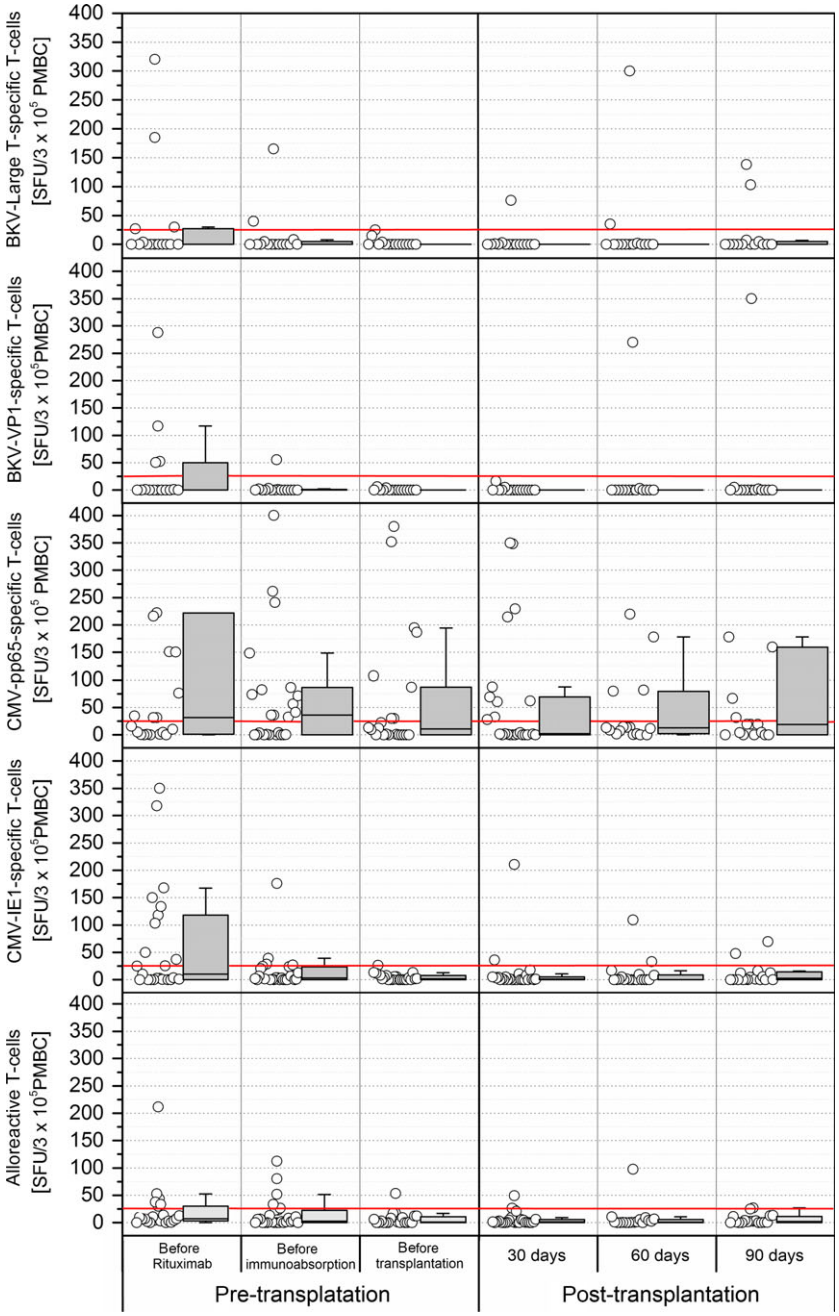

Figure 4 Decreasing BKV-, CMV-specific T cells, and alloreactivity along the course of ABO desensitization.

T cells prior to transplantation (Fig. 5b). cABO-KTRs developing BK viremia showed stable or increasing frequencies of BKV-specific T cells directed to BKV-VP1 and BKV large T ($P < 0.05$). Three of 25 cABO-KTRs (12%) without BKV-specific immunity directed to BKV large T and/or BKV-VP1 developed early onset BK viremia.

Alloreactivity

Six of 24 iABO-KTRs (25%) showed well-detectable alloreactive T cells prior to ABO desensitization (Fig. 5c). Cellular alloreactivity after transplantation was low or undetectable with only 2 of 24 iABO-KTRs (8%) showing alloreactive T cells. Only 1 iABO-KTR with initially high frequencies of alloreactive T cells showed detectable alloreactive T cells. One of 6 KTRs (17%) with initially detectable alloreactive T cells showed repeated acute rejection episodes of Banff IB and IIA in the early period after transplantation.

In comparison, 5 of 27 cABO-KTRs (19%) showed detectable alloreactive T cells prior to transplantation. After transplantation, 7 KTRs (26%) showed alloreactive T cells

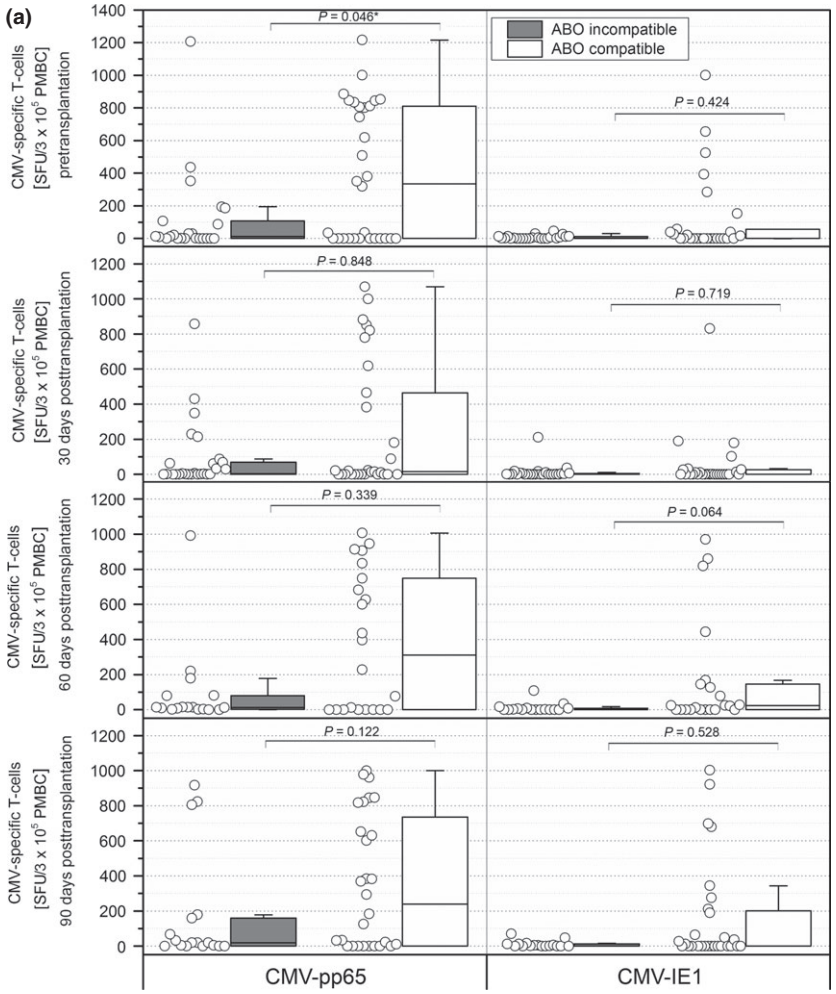

Figure 5 (a,b) Impaired CMV- and BKV-specific immunity in patients undergoing ABO desensitization. 5C Decrease in alloreactive T cells among patients undergoing ABO desensitization. KTRs undergoing ABO desensitization show significantly lower alloreactive T cells in the early post-transplant period compared to cABO-KTRs.

with high frequencies ranging from 128 to 575 spot-forming units per 3.0 $\times$ 10^5 PBMC in 4 KTRs (15%). Four of 7 cABO-KTRs (57%) with detectable alloreactive T cells showed acute rejection episodes in the early period after transplantation. Two of 7 KTRs (29%) showed repeated severe acute rejection episodes of Banff IIA and IIB in the early period after transplantation.

iABO-KTRs showed significantly lower alloreactive T cells compared to cABO-KTRs at +30 and +60 days ($P < 0.05$), and a tendency for lower alloreactive T cells at +90 days ($P = 0.056$).

Discussion

A continuous immune control is required to prevent reactivation of latent viruses, bacterial and fungal infections after renal transplantation. With regard to adaptive immunity, T cells are of central importance in controlling infectious complications [17–22]. Using ABO desensitization with rituximab, immunoadsorption, and IVIG our results show favorable intermediate-term outcomes for iABO transplantation in line with previous observations [5,23–25]. Despite lower rates of acute cellular rejections, comparable tacrolimus trough levels and MMF dosing, we noted a marked increase in the number of infectious complications in iABO-KTRs. These data are in agreement with recent studies showing an increased incidence of infections after iABO transplantation [9–11,26]. Therefore, we hypothesized that a severe immunocompromised state due to ABO desensitization renders iABO-KTRs more susceptible for infections early after transplantation.

Firstly, concerning the impact of ABO desensitization on overall cellular immunity, our observations suggest overimmunosuppression with severe CD4+ lymphopenia plus

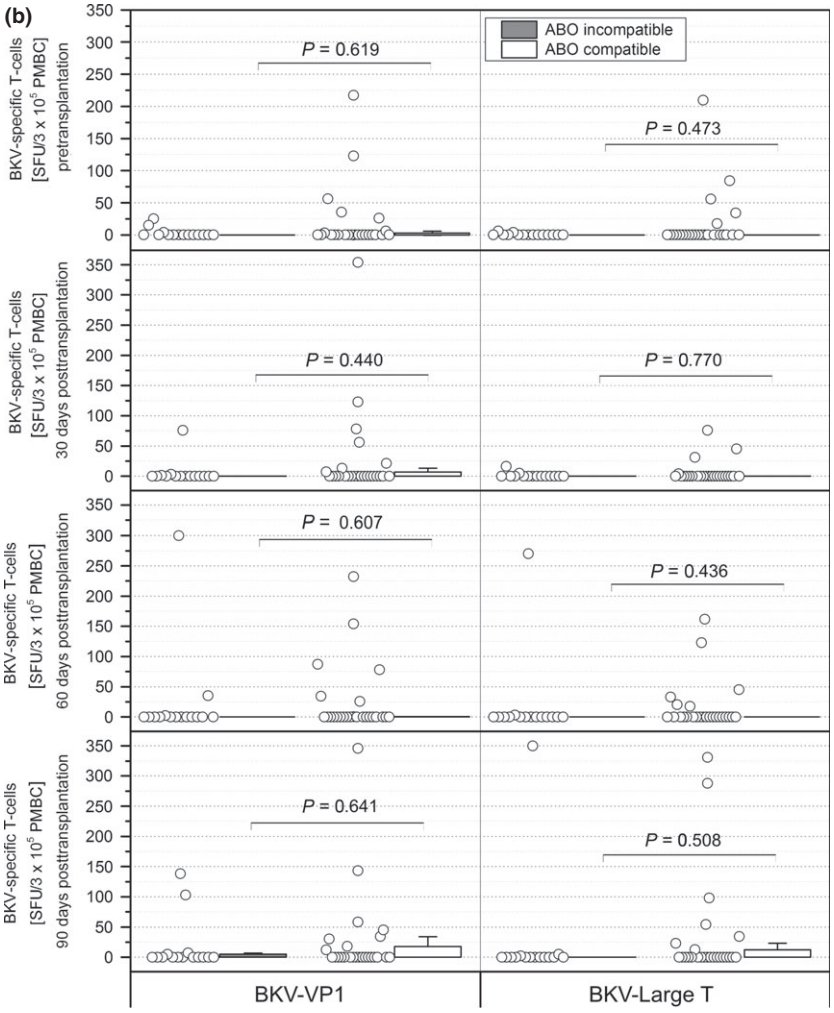

Figure 5 Continued

decreased IFNγ serum levels as the most important risk factor for the increased incidence of infections. Besides the impact of ABO desensitization on T-cell number, however, our study was not able to identify specific cellular phenotypes or functional differences between the different groups. Rituximab-mediated depletion of B cells is expected to result in poor antibody responses to antigens. While few B cells remain after administration of rituximab and show a memory phenotype, returning B cells are mainly naïve, indicating *de novo* production of B cells. Therefore, serum levels of IgG remain unchanged, while decreases in IgM and IgE were observed [27–30]. Besides their role in antibody synthesis, it may be speculated that the decrease of B cells as antigen-presenting cells induces a decrease in activated T cells [13–15,28]. Rituximab-mediated B-cell depletion down-regulates co-stimulatory molecules, CD40 and CD80 on B cells, thus affecting T-helper-cell activation with abnormalities in cytokine production.

There is some evidence that the B-cell depletion induced by rituximab may result in decreased numbers preferentially of CD4+ T cells and may interfere with T-cell function [25,27]. As small numbers of T cells and NK cells express CD20, rituximab also mediates a direct effect on cellular immunity with decreased numbers of T- and NK cells. Changes in NK, macrophage, and or dendritic cell functions have also been reported during B-cell depletion but require further investigation. Although hypogammaglobulinemia has been associated with infection, hypogammaglobulinemia is uncommon after single administration of rituximab in particular in the setting of ABO desensitization with administration of IVIG.

Previous work showed that IVIG inhibits CD4+ T-cell activation and proliferation due to intracellular interference of IVIG with presentation of native antigens by antigen-presenting cells [31–33]. IVIG leads to a decreased internalization inside antigen-presenting cells with a reduced

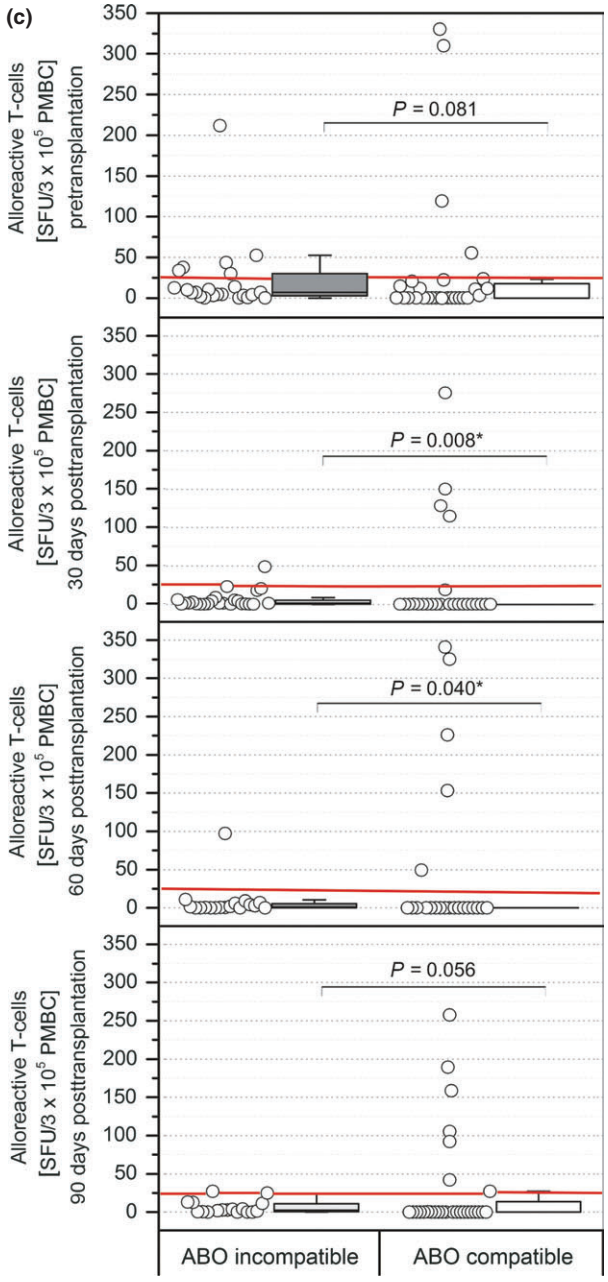

Figure 5 Continued

amount of antigen presented by MHC II molecules to CD4+ T cells. In addition to this inhibition of CD4+ T cells, it has been demonstrated that antigen-specific CD8+ T-cell activation after cross-presentation of immune complexes is strongly reduced in the presence of therapeutic doses of IVIG [31–33]. Recent work showed that IVIG contains antibodies specific to a highly conserved portion of human HLA class I antigens and that these antibodies were able to inhibit class I-restricted T-cell-mediated cytotoxicity [34].

Secondly, concerning the impact of ABO desensitization on virus-specific immunity, our results suggest impairment of CMV- and BKV-specific immunity in iABO-KTRs associated with increased incidences of viral reactivation. Previous work showed that early reconstitution of virus-specific cellular immunity prevents or reduces the duration of CMV- and BK viremia [12–17,19,32,33]. This study confirms that iABO-KTRs undergoing ABO desensitization develop a more severe immunocompromised state

compared to cABO-KTRs resulting in a decline of BKV- and CMV-specific T cells insufficient to regulate viral replication. Here, iABO-KTRs particularly show impaired protective immunity directed to CMV-IE1 and BKV large T [18,19,21,35,36]. Our results further indicate that highly increased rates of CMV replication in iABO-KTRs serve as an important cofactor to the progression of bacterial and fungal infection [20,37,38]. Mechanisms that contribute to these effects include neutropenia or immune effects as immune suppression or graft rejection which might necessitate a further increase in immunosuppressive therapy [28,35–37]. The unexpected reduction of serum IL-10 levels at +3 months may reflect the increased incidence of CMV viremia in iABO-KTRs with a peak at +2 months. Here, significantly decreased IL-10 levels have been described in KTRs recovering from CMV viremia [31]. Our data further suggest that the increased incidence of KTRs with BK viremia progressing to BKV-associated nephropathy results from impaired cellular immunity not able to expand sufficient BKV-specific T cells [18,21]. Although there is a tendency for higher BK viremia rates among iABO-KTRs, our data support previous observations of an increased risk of progression to BKV-associated nephropathy [9]. The lack of EBV viremia in iABO-KTRs may be attributed to the use of rituximab in ABO desensitization. Rituximab-mediated B-cell depletion results in both, elimination of EBV-infected B cells of the recipient, and prevention from donor-derived EBV-infection.

Thirdly, concerning the impact of ABO desensitization on alloreactivity, our results suggest that ABO desensitization may have significant effects on augmentation of alloreactive T cells. It has been reported previously that administration of rituximab may decrease preformed anti-HLA alloantibody titers in presensitized KTRs [39]. Suggested inhibition of T-cell proliferation and function by ABO desensitization may contribute to these observations and support favorable allograft outcome with lower rates of acute cellular rejection and inhibition of progressive immune-mediated graft injury due to alloreactive T-cell immunity.

The hypothesis of previous works [9] that the increased incidence of infectious complications is related to alterations of the immune system due to blood group incompatibility and the occurrence of a graft accommodation phenotype are not supported by this study. Although poor HLA-match did not reach significance as an independent risk factor for infectious complications, as shown previously [40], more intensified immunosuppression due to an increased risk of acute allograft rejection may lead to a further immunocompromised state.

Our findings suggest that the current protocol does carry an enhanced risk of over-immunosuppression. Our results reveal the complexity of mechanisms whereby rituximab, IVIG, and maintenance immunosuppression exert its effects, mainly those that go beyond simple B-cell depletion. When and in which situation each of these mechanisms is dominant should be the subject of future studies. A previous study demonstrated that ABO desensitization using plasmapheresis and IVIG may be equally effective compared to ABO desensitization using rituximab, plasmapheresis, and IVIG concerning allograft outcome. Very recent work suggested tailoring the use of rituximab according to initial ABO blood group antibody titers with comparable results [41,42].

Authorship

TS: participated in data collection, writing of the paper, performance of the research, and data analysis. MS: participated in the performance of the research. PR: participated in research design, writing of the paper, performance of the research, and data analysis.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Patient characteristics: ABO compatible (cABO) versus ABO incompatible (iABO).

Table S2. Infectious complications: ABO compatible (cABO) versus ABO incompatible (iABO).

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