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Alemtuzumab Induction and Delayed Acute Rejection in Steroid-Free Simultaneous Pancreas-Kidney Transplant Recipients

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Background. The optimal immunosuppressive regimen in simultaneous pancreas-kidney transplant (SPKT) recipients that prevents acute rejection episodes (AREs) and allows optimal outcome remains elusive. **Methods.** This cohort study assessed incidence and time to AREs in 73 consecutive SPKT recipients receiving alemtuzumab induction and steroid-free maintenance with tacrolimus and mycophenolate mofetil. A cohort with single high-dose antithymocyte globulin (ATG; n = 85) and triple therapy served as controls. In addition, we provided mechanistic insights in AREs after alemtuzumab depletion, including composition and alloreactivity of lymphocytes (flow cytometry and mixed lymphocyte reaction) plasma alemtuzumab levels (enzyme-linked immunosorbent assay), and maintenance drug exposure. **Results.** Overall number of AREs at 3 years was significantly lower with alemtuzumab versus ATG induction (26.0% vs 43.5%; adjusted hazard ratio, 0.38; $P = 0.029$). Most AREs (94.6%) with ATG occurred within the first month, whereas 84.2% of AREs with alemtuzumab occurred beyond 3 months. Patients with and without an ARE in the steroid-free alemtuzumab group showed no differences in composition of lymphocytes, or in alemtuzumab levels. Of note, more than two thirds of these AREs were preceded by empiric tacrolimus and/or mycophenolate mofetil dose adjustments due to viral infections, leukopenia, or gastrointestinal symptoms. **Conclusions.** Alemtuzumab induction resulted in a significant lower incidence of AREs. Empiric dose adjustments beyond 3 months in the absence of steroids carry a significant risk for subsequent rejection in SPKT recipients.

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In patients with type I diabetes mellitus and end-stage renal disease, simultaneous pancreas-kidney transplantation (SPKT) is the preferred treatment option. SPKT results in a significant survival benefit,¹ due to restoration of renal function and normalization of blood glucose levels with stabilization of microvascular and macrovascular complications.^{2,3} A

relative drawback of SPKT is that these patients experience an acute rejection episode (ARE) more frequently compared with recipients of a kidney transplant alone. This is most likely related to a combination of factors, including the lack of prospective HLA matching, the higher antigenic load of the combined procedure, diabetic gastropathy with

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J.R.B. designed the study, collected and analyzed the data and wrote the article. S.H. performed experiments (functional immunological assays) and analyzed the data. D.J. A.R.M. analyzed the data of the alemtuzumab plasma levels. D.L.R. performed experiments (functional immunological assays). M.J.K.M. and P.J.M.v.d.B. collected the clinical data. M.V. performed experiments (functional immunological assays). C. M.J.v.d.Z. performed experiments (alemtuzumab plasma levels). R.G.B. analyzed the

data of alemtuzumab plasma levels. A.E.B. and J.R. collected the clinical data. M.J. D.v.T. performed experiments (alemtuzumab plasma levels) and analyzed the data. F.H.J.C. analyzed the data of functional immunological assays. M.E.J.R. and J.W.d. F. designed the study, analyzed the data, and reviewed the article.

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unpredictable drug absorption profiles, and an altered immune response in the context of autoimmunity.⁴⁻⁷

Induction therapy is the cornerstone of contemporary immunosuppression in renal transplantation and SPKT in particular.^{6,8-10} In renal transplantation, the type of induction therapy may be chosen based on the recipient's risk of rejection and/or delayed graft function. Recently, large prospective studies have shown lower numbers of AREs after induction with alemtuzumab as compared with basiliximab.^{11,12} In high-risk transplant recipients, alemtuzumab was as effective as antithymocyte globulin (ATG),¹² resulting in increasing numbers of patients receiving alemtuzumab induction therapy.

More than 80% of SPKT recipients in the United States receive induction therapy with depleting antibodies.^{4,10,13,14} The efficacy of alemtuzumab in SPKT is less well documented, mainly due to the limited number of patients studied.¹⁵⁻¹⁹ Two studies showed comparable numbers of AREs after induction with alemtuzumab and ATG,^{16,19} 2 others showed a trend toward lower rejection rates with alemtuzumab.^{17,18} More detailed information on timing of AREs and mechanistic insights in AREs have not been published so far.

In our cohort of SPKT recipients, we compared the incidence and timing of AREs of patients receiving induction therapy with alemtuzumab with those receiving ATG. In addition, we provided mechanistic insights in AREs after alemtuzumab induction, including composition and alloreactivity of lymphocytes at time of rejection, plasma levels of tacrolimus (TAC) and mycophenolate mofetil (MMF), and dose adjustments made by the treating physicians due to adverse events. Finally, we have also analyzed plasma alemtuzumab levels at several time points after transplantation in relation to AREs.

MATERIALS AND METHODS

Study Population and Immunosuppression

This is a nonrandomized, single-center cohort study in which all consecutive SPKT recipients receiving induction therapy with a depleting antibody between June 2002 and

December 2012 at the Leiden University Medical Center were included (n = 165) (Figure 1). Before November 2007, the standard induction regime consisted of a single high-dose of ATG-Fresenius (9 mg/kg, intravenously (i.v.) on day 0, before reperfusion), as described previously.⁶ After this date, patients were treated according to a new standard therapy with alemtuzumab induction (15 mg, subcutaneously (s.c.) on day 0 before surgery, and 15 mg s.c. on day 1). Maintenance immunosuppression was started on day 0 and consisted of TAC (Prograft twice daily, trough 8-12 µg/L first 6 weeks, 6-9 µg/L thereafter) and MMF (750 mg twice daily, 12 hours area under the curve (AUC₁₂) 30-45 mg h⁻¹ L⁻¹). Patients using cyclosporine (n = 7) as maintenance therapy were excluded from the current analysis (Figure 1). In case of ATG induction, patients remained on low-dose corticosteroids (CS).⁶ In patients with alemtuzumab induction a 3-day course of methylprednisolone was given (500 mg during surgery, 250 mg on day 1, 125 mg on day 2) and on day 3 CS were completely stopped. Pancreatic duct management was different for patients in the ATG and alemtuzumab group; before 2008, bladder drainage was the most common method of pancreatic duct management and since 2008 direct enteric drainage.

Prophylaxis

All patients received cotrimoxazol for 6 months as pneumocystis jiroveci pneumonia prophylaxis. Patients transplanted from 2006 onward also received valganciclovir prophylaxis for a minimum of 3 months, except those with a cytomegalovirus (CMV)-negative donor and recipient status. Before 2006, a preemptive strategy for CMV was used.²⁰ CMV, Epstein-Barr virus (EBV) and from 2007 also BK virus (BKV) load were screened routinely using specific quantitative polymerase chain reaction.

Graft Loss and Rejection

Kidney graft failure was defined as allograft removal or loss of renal function requiring dialysis. Pancreas graft failure

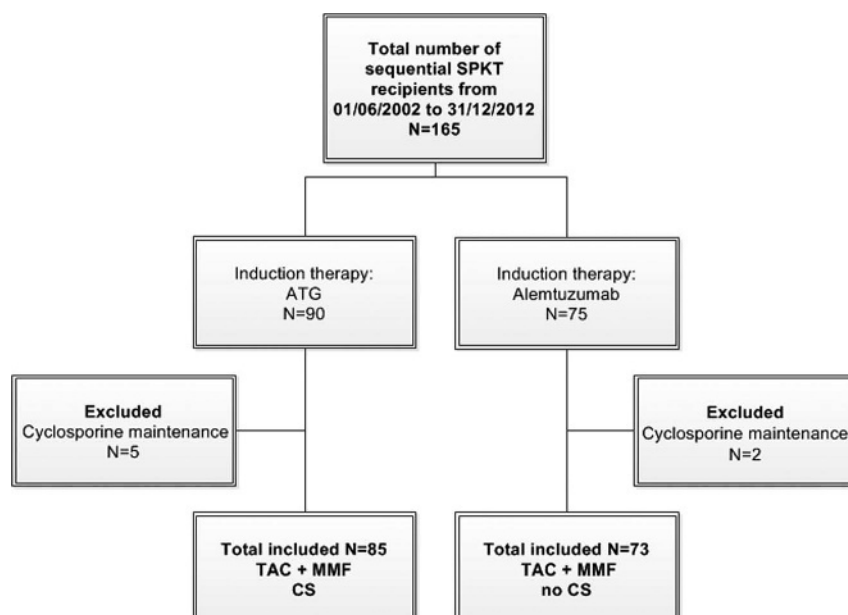


FIGURE 1. Patient distribution. Cohort of subsequent SPKT recipients receiving induction therapy with a depleting antibody between June 2002 and December 2012.

was defined as allograft removal or loss of endocrine pancreatic function requiring exogenous insulin therapy.

All clinically suspected and treated AREs were confirmed by histology. In case a percutaneous kidney biopsy was contraindicated, the episode was classified by the function response to high-dose CS and/or depleting antibodies, while excluding inadequate systemic drug exposure. For the diagnosis of pancreas allograft rejection, the clinical definition was used: increase of serum amylase and hyperglycemia, in absence of other explanations including imaging by CT scan. Pancreatic biopsies were not performed. AREs were treated with methylprednisolone (1000 mg per day for 3 consecutive days), or cell-depleting antibodies (ATG or alemtuzumab) in case of steroid-resistant rejections. Antibody-mediated rejection was treated with a combination of methylprednisolone, cell-depleting antibody, i.v. immunoglobulins and plasma exchange when donor specific antibodies (DSAs) were present in the circulation.

Functional Immunological Assays

Immunophenotyping and functional assays were performed to investigate repopulation and function of immune cells in 19 patients with alemtuzumab induction (ARE n = 6, no ARE n = 13) and in 11 patients with ATG (ARE n = 5, no ARE n = 6). Of the patients with an ARE available frozen blood samples were collected at time of rejection (before ARE therapy). Patients without an ARE were matched for timing. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Hypaque (Hospital Pharmacy LUMC, Leiden, the Netherlands) density centrifugation and frozen in liquid nitrogen until further use. Upon thawing flow cytometry was performed according to standard protocols (SDC, **Materials and Methods**, <http://links.lww.com/TXD/A33>).

One-way mixed lymphocyte reactions were performed with recipient PBMC against irradiated donor or third party spleen cells (1×10^5 cells per well, triplicate), as previously described.²¹ In case there were no spleen cells available, irradiated donor and third party PBMCs were used. Results are expressed as mean counts per minute of triplicate wells (SDC, **Materials and Methods**, <http://links.lww.com/TXD/A33>).

At 6 months posttransplantation, patient sera of the alemtuzumab group were screened for the presence of HLA-specific antibodies by complement-dependent cytotoxicity using a cell panel of 54 healthy donors. Sera were also screened for antibodies against HLA class I and II using enzyme-linked immunosorbent assay (LAT-M; One Lambda, Inc., Canoga Park, CA). All samples that tested positive were further specified with Luminex single antigen test (LABScreen SA, One Lambda, Inc.).

Alemtuzumab Plasma Levels

In 10 patients with an ARE within 6 months and in 10 age- and sex-matched patients without an ARE, alemtuzumab plasma levels were analyzed at 3 time points (range, 13-99 days posttransplantation). Alemtuzumab plasma levels were determined using an enzyme-linked immunosorbent assay (lower limit of detection 0.01 µg/mL) (SDC, **Materials and Methods**, <http://links.lww.com/TXD/A33>).

Statistical Analysis

Descriptive analyses with proportions, means and standard deviations were used to describe the characteristics at baseline. Univariate analysis was performed using the unpaired

2-tailed *t* test for continuous variables and χ^2 for categorical variables. To compare AREs between the ATG and alemtuzumab group Cox regression analysis was performed to control for potential confounders. To compare plasma alemtuzumab levels for patients with and without an ARE, Kaplan-Meier estimates (log rank) were used. A *P* value less than 0.05 was considered statistically significant. Analyses were performed using SPSS, version 23.0 and GraphPad Prism version 6.0.

RESULTS

A total of 158 SPKT recipients were identified, 85 patients treated with ATG and 73 patients treated with alemtuzumab induction. Baseline recipient, donor, and transplant characteristics are summarized in Table 1. There were no significant differences in baseline characteristics, except for more patients receiving preemptive transplants and primary enteric duct drainage in recent years with alemtuzumab induction (*P* = 0.01 and *P* < 0.001, respectively). Compared with alemtuzumab, the ATG group had longer cold ischemia times for both the kidney and the pancreas (Table 1).

Lower Overall Incidence and Delayed Onset of AREs After Alemtuzumab Induction

After 3 years of follow-up, the overall number of AREs was significantly lower in patients who received alemtuzumab compared with patients with ATG induction (Figure 2), with

TABLE 1.
Patients characteristics

N	ATG	Alemtuzumab	P
	85	73	
Recipient			
Age, y	41.9 ± 7.5	44.1 ± 8.6	0.091
Sex: male, %	56.5	63.0	0.404
Diabetes duration, y	29.9 ± 7.5	29.4 ± 8.0	0.709
HbA1c level, %	8.9 ± 2.0	8.7 ± 2.1	0.560
Preemptive transplant, %	31.8	52.1	0.010
Donor			
Age, y	32.8 ± 12.5	36.0 ± 12.8	0.118
Sex: male, %	47.1	53.4	0.425
Transplant			
HLA-mismatch class I	2.9 ± 1.0	2.9 ± 0.8	0.960
HLA-mismatch class II	1.4 ± 0.6	1.5 ± 0.6	0.633
Max PRA, mean	5.4 ± 11.1	3.8 ± 8.4	0.307
Cold ischemia time kidney, h	11.6 ± 2.5	10.0 ± 2.9	0.001
Cold ischemia time pancreas, h	12.6 ± 2.6	10.7 ± 1.9	<0.001
Total warm ischemia time kidney, min	28.7 ± 8.0	29.3 ± 6.4	0.594
Total warm ischemia time pancreas, min	27.8 ± 7.4	28.5 ± 6.6	0.531
Delayed graft function kidney, %	4.7	9.6	0.229
Repeat transplant, %	1.2	1.4	0.914
Primary drainage, enteric %	23.5	72.6	<0.001
CMV IgG status			
D+/R+, N	14	20	0.096
D+/R-, N	19	12	0.351
D-/R+, N	19	15	0.783
EBV IgG status			
R-, N	14	10	0.606

All data are mean ± SD, unless otherwise specified.

PRA, panel-reactive antibody.

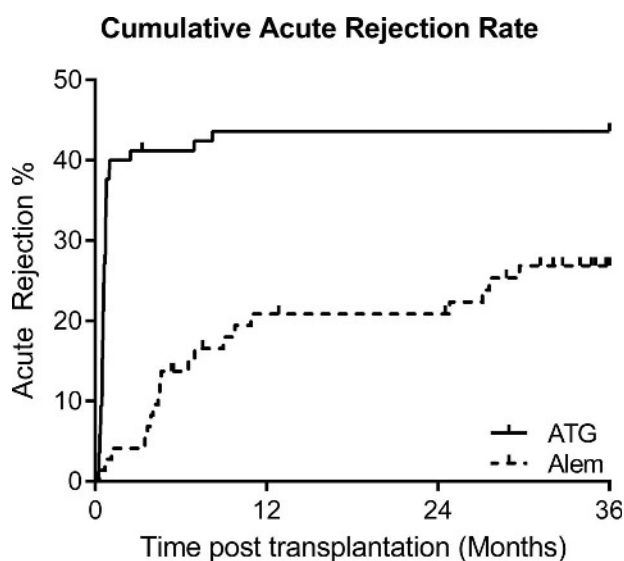


FIGURE 2. Alemtuzumab induction was associated with a lower overall incidence, but delayed onset of AREs. In the ATG group 94.6% of the AREs occurred within the first 3 months post transplant, while after alemtuzumab induction 15.8% of the AREs occurred within 3 months, 63.2% between 3 and 12 months, and 21.0% beyond 1 year. Alem, alemtuzumab.

an adjusted hazard ratio of 0.38 and 95% confidence interval of 0.16 to 0.91 ($P = 0.029$) (Table S1, SDC, <http://links.lww.com/TXD/A33>). In total, 37 AREs were documented in the ATG group (43.6%), and 19 with alemtuzumab induction (26.0%). In the alemtuzumab group, all AREs were confirmed by biopsy, in 7 patients of the ATG group biopsies were not performed (oral anticoagulants [$n = 3$], logistic reasons [$n = 4$]). Three patients (ATG [$n = 2$]; alemtuzumab [$n = 1$]) were clinically diagnosed with pancreas allograft rejection, without rejection of the kidney. Steroid resistance AREs were seen in 17 patients (45.9%) with ATG and in 9 patients (47.4%) with alemtuzumab induction (data not shown). Two patients had an antibody-mediated rejection (ATG [$n = 1$], alemtuzumab [$n = 1$]) with C4d-positive staining and detectable DSAs in 1 patient. Six months posttransplantation DSAs were determined in all patients in the alemtuzumab group, but in only 1 patient de novo DSAs were found.

In the ATG group, 94.6% of the AREs occurred within the first 3 months after transplantation, the remaining 5.4% between 3 and 12 months, and none thereafter. In the alemtuzumab group, however, 15.8% of the AREs occurred in the first 3 months, 63.2% between 3 and 12 months, and 21.0% between 12 and 36 months (Figure 2). Thus, in the alemtuzumab group AREs were delayed, with a peak incidence between 3 and 12 months after transplantation.

No Difference in Survival, Graft Function and Metabolic Parameters

Mean 3-year patient survival rate was 93.7% with no difference between both groups, also after correcting for potential confounders (Table S2, SDC, <http://links.lww.com/TXD/A33>). During the first 3 years of follow-up, 5 (5.9%) of 85 patients died in the ATG group (sepsis [$n = 1$], respiratory insufficiency [$n = 2$], unknown cause [$n = 2$]) and 5 (6.8%) of 73 patients in the alemtuzumab group

(aspiration during hepatic coma [$n = 1$], hemorrhagic shock [$n = 1$], unknown cause [$n = 3$]). Five patients were lost to follow-up (ATG [$n = 2$], alemtuzumab [$n = 3$]). Death-censored kidney graft survival at 3 years was 95.2% and 95.4% with ATG and alemtuzumab, respectively. More patients in the ATG group lost their pancreas due to thrombosis (9 vs 2, resulting in a lower pancreas survival rate with ATG (83.2%) compared with alemtuzumab (92.7%; adjusted hazard ratio, 0.18; 95% confidence interval, 0.31-1.00; $P = 0.051$) (Table S2, SDC, <http://links.lww.com/TXD/A33>).

Kidney allograft function at 1- and 3-year posttransplant defined by creatinine or endogenous creatinine clearance was comparable in both groups, and there was no difference in the degree of proteinuria (Table 2). Pancreas allograft function, defined by C-peptide and glycosylated hemoglobin (HbA1c), was also comparable for the ATG and alemtuzumab group (Table 2).

Because CS are well-known contributors to various cardiovascular risk factors, the potential benefit of a steroid-free regime on lipids was analyzed. Total cholesterol levels were not different for patients with ATG and maintenance CS and patients with alemtuzumab and a steroid-free regime at 1 year (4.7 ± 0.8 and 4.9 ± 1.3 , respectively) and at 3 years posttransplantation (4.7 ± 1.0 and 4.6 ± 0.8 , respectively) (data not shown).

TABLE 2.
Graft function and infectious complications

N	ATG	Alemtuzumab	P
	85	73	
Kidney function			
Creatinine, $\mu\text{mol/L}$			
1 y	120.3 \pm 35.4	120.9 \pm 30.1	0.907
3 y	124.9 \pm 46.4	133.6 \pm 39.6	0.242
Proteinuria, g/24 h			
1 y	0.4 \pm 0.4	0.4 \pm 0.5	0.857
3 y	0.4 \pm 0.5	0.3 \pm 0.3	0.583
Endogenous creatinine clearance, mL/min			
1 y	69.6 \pm 24.2	69.6 \pm 23.9	0.988
3 y	69.0 \pm 31.0	63.8 \pm 22.0	0.272
Pancreas function			
C-peptide, nmol/L			
1 y	1.9 \pm 1.0	1.7 \pm 1.5	0.541
3 y	1.6 \pm 1.4	1.5 \pm 1.5	0.854
HbA1c level, %			
1 y	5.5 \pm 0.7	5.4 \pm 0.5	0.398
3 y	5.4 \pm 0.5	5.4 \pm 0.4	0.801
Infections			
CMV infection, N			
Primary, N	14	11	0.810
Primary under prophylactic therapy, N (%)	5/9 (55.6)	11/12 (91.7)	0.055
Reactivation, N	4	2	0.519
EBV infection, N	3	3	0.849
Wound infections, N	7	5	0.743
Abdominal infections, N	20	12	0.269
Pulmonary tract infections, N	8	4	0.245
Urinary tract infections, N			
Total	68	36	0.001
With enteric drainage, N (%)	10/20 (50.0)	23/53 (43.4)	0.729

All data are mean \pm SD, unless otherwise specified.

No Difference in Infectious Complications

The occurrence of wound infections, pulmonary tract infections and abdominal abscesses was comparable for both groups (Table 2). Urinary tract infections were more frequently seen after ATG induction ($P = 0.001$) and were related to more frequent use of exocrine pancreatic bladder drainage in that period. The subgroup of patients with enteric drainage did not have a higher incidence (Table 2).

The occurrence of (opportunistic) viral infections, including CMV, EBV, herpes simplex, and varicella zoster virus was also comparable between the ATG and alemtuzumab group (Table 2). Overall, 15.8% of the SPKT recipients suffered from a primary CMV infection. Of the 21 patients (ATG $n = 9$, alemtuzumab $n = 12$) with a D+/R- serostatus and prophylactic therapy 16 patients (ATG $n = 5$, alemtuzumab $n = 11$) experienced a primary CMV infection. CMV reactivation was found in 13.3% of the patients, and this did not differ between ATG ($n = 4$) and alemtuzumab ($n = 2$) (Table 2). An EBV infection was seen in 6 patients (ATG $n = 3$, alemtuzumab $n = 3$), of which 1 patient (ATG) developed a posttransplant lymphoproliferative disorder. In the alemtuzumab group 23.3% had a BKV infection. This could not be compared retrospectively with the ATG group.

Late AREs After Alemtuzumab Were Not Associated With Differences in Repopulation of Lymphocyte Subsets

Because in the alemtuzumab group AREs mainly occurred beyond 3 months posttransplantation, we investigated

whether this could be explained by preferential repopulation of lymphocyte subsets. Flow cytometry analysis of patients with alemtuzumab induction did not reveal differences in the percentage of specific T cell subsets between patients with and without an ARE, including $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells (Figures 3A-B). This also applied to $CD4^+CD25^{hi}CD127^{low}$ regulatory T cells (Figure S1A, SDC, <http://links.lww.com/TXD/A33>). The percentage of $CD19^+$ B cells was also comparable in both groups (Figure 3C), as well as transitional B cells ($CD19^+CD24^{hi}CD38^{hi}$), which have been described to be enriched for Bregs²² (Figure S1B, SDC, <http://links.lww.com/TXD/A33>). Within the ATG group, there was also no difference found in the abovementioned lymphocyte subsets between patients with an ARE and those without.

In both the alemtuzumab and ATG group, the extent of T cell proliferation towards the donor or third party was comparable between patients at time of rejection and nonrejecting patients (Figure 3D). Interestingly, patients experiencing an ARE did not show an increased donor-specific response compared to 3rd party. In line with these data, no differences in $IFN\gamma$ production were found in the supernatants of these cultures (data not shown).

Late AREs Were Not Associated With Plasma Alemtuzumab Levels

Plasma alemtuzumab levels showed high interpatient variability, ranging from 0.04 to 0.30 $\mu\text{g/mL}$ at 30 days (± 7 days) and from undetectable ($<0.01 \mu\text{g/mL}$) to 0.08 $\mu\text{g/mL}$ at 90 days (± 7 days) (Figure 4A). The levels measured over time

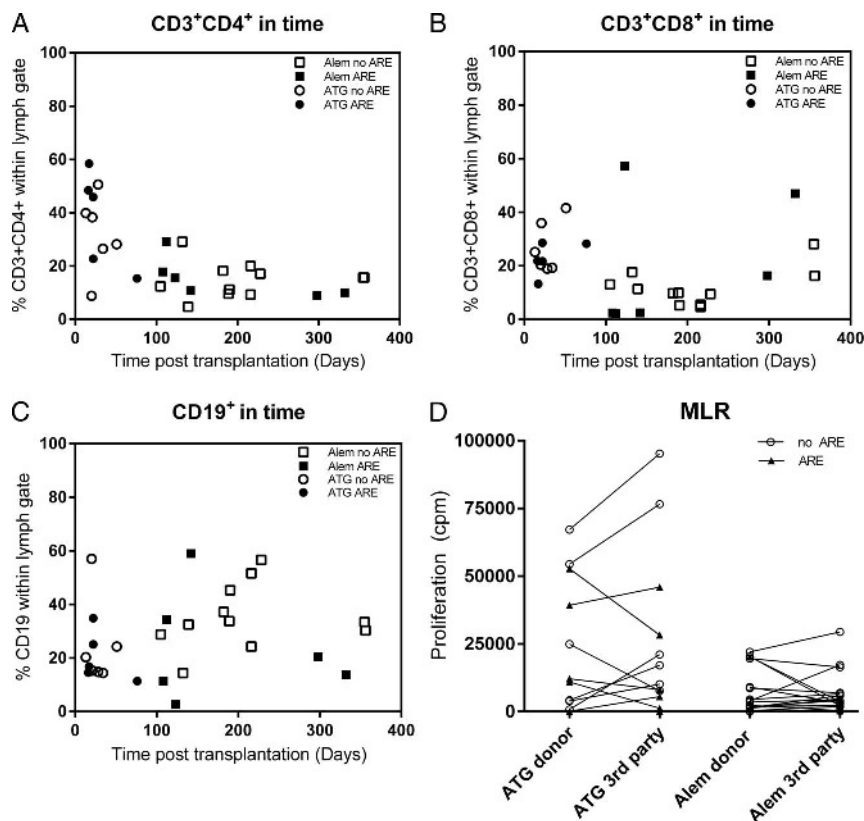


FIGURE 3. Late AREs after alemtuzumab induction were not associated with differences in repopulation of lymphocyte subsets. Phenotypic and functional analysis of lymphocytes from patients with and without an ARE, after ATG or alemtuzumab induction therapy. A, Percentage of $CD3^+CD4^+$. B, Percentage of $CD3^+CD8^+$. C, Percentage of $CD19^+$. D, MLR assays showing no difference in proliferation to donor antigens and third-party antigens between patients with and without an ARE. Alem, alemtuzumab; cpm, counts per minute; MLR, mixed lymphocyte reaction.

varied per recipient; some had a high peak and then a rapid decline, whereas others had a flatter curve (data not shown). In 13 of 20 patients (ARE $n = 7$, no ARE $n = 6$) alemtuzumab levels were no longer detectable at 3 months. The kinetics of decay of the alemtuzumab levels were not different for patients with and without an ARE (log-rank 0.486) (Figure 4B), also when cut-off values for alemtuzumab of less than 0.03 $\mu\text{g/mL}$, less than 0.05 $\mu\text{g/mL}$, and less than 0.10 $\mu\text{g/mL}$ were applied (data not shown).

Late AREs After Alemtuzumab Were Preceded by Empiric Dose Reductions

Patients with alemtuzumab induction and an ARE had similar pretransplant panel-reactive antibody levels (6.0 ± 14.9 vs 3.0 ± 4.3) and HLA mismatch degree (2.7 ± 0.9 vs 2.9 ± 0.8 for class I; 1.3 ± 0.6 vs 1.5 ± 0.6 for class II) compared to patients without an ARE (data not shown).

Subsequently, plasma levels and adjustments in maintenance immunosuppression were investigated. TAC trough levels were 7.6 ± 1.9 $\mu\text{g/L}$ and 8.7 ± 2.5 $\mu\text{g/L}$ at 3 months posttransplantation and 6.6 ± 1.5 $\mu\text{g/L}$ and 7.2 ± 1.8 $\mu\text{g/L}$ at 6 months posttransplantation for patients with and without an ARE, respectively ($P = 0.123$ and $P = 0.296$, data not shown). Total daily MMF dose was also comparable at

month 3 (1536 ± 536 mg and 1448 ± 416 mg) and month 6 (1273 ± 467 mg and 1304 ± 441 mg) in patients with and without an ARE, respectively ($P = 0.518$ and $P = 0.833$, data not shown).

Within the first year after transplantation, 84.9% remained steroid-free. Eleven patients were switched to a regime with CS most commonly following an ARE ($n = 4$). The other patients suffered from side effects related to TAC ($n = 2$) or MMF ($n = 2$) or were fast metabolizers ($n = 3$, all with CYP3A5 *1/*3 genotype).

Of interest, in the alemtuzumab group 13 (68.4%) of the 19 AREs were preceded by dose adjustments or low exposure to immunosuppression. In contrast, with ATG this was noted only in 3 (8.1%) of 37 patients. Of these 13 patients in the alemtuzumab group, the treating physicians reduced the dose of TAC and/or MMF in 7 patients; 5 patients with viral infections (BKV $n = 4$, BKV + CMV $n = 1$) resulting in relative low TAC levels (mean 4.3 $\mu\text{g/L}$) and low daily MMF dose (mean 300 mg twice daily) before the ARE, and 2 patients with leukopenia ($1.87 \times 10^9/\text{L}$ and $2.40 \times 10^9/\text{L}$) resulting in low MMF exposure (AUC_{12} of 20 and 33 $\text{mg h}^{-1} \text{L}^{-1}$ at a dose of 500 mg twice daily and 250 mg twice daily, respectively) before the ARE. Furthermore, 1 patient suffered from vomiting, 3 patients had low exposure to immunosuppressives for a longer period early posttransplantation (TAC [$n = 2$], trough levels 4.7 and 5.5 $\mu\text{g/L}$, MMF [$n = 1$] AUC_{12} 17 $\text{mg h}^{-1} \text{L}^{-1}$). In addition, in 2 patients, a change in immunosuppression had taken place (1 patient switched to cyclosporine and back to TAC again, another from MMF to azathioprine). In the remaining 6 patients with AREs, no apparent cause could be identified (Figure 5: patient-example).

DISCUSSION

The current cohort study assessed the incidence and timing of AREs in SPKT recipients receiving alemtuzumab 2 times 15 mg s.c. followed by steroid-free maintenance and compared the results to a cohort receiving ATG induction and triple maintenance with TAC, MMF and CS. Furthermore, mechanistic insights in AREs after alemtuzumab induction were provided, including repopulation and alloreactivity of lymphocytes, plasma alemtuzumab levels and exposure to maintenance drugs.

Our study demonstrated that alemtuzumab induction in SPKT was associated with significant lower overall incidence of AREs as compared to patients who received ATG. AREs with alemtuzumab induction occurred later in time, with a peak incidence beyond the first 3 months postautopropter empiric dose reductions according to protocol. In contrast, with a single high-dose administration of ATG almost all AREs occurred within the first month. Patients with an ARE beyond month 3 in the alemtuzumab group showed no differences in repopulation of their T and/or B cells or decline of plasma alemtuzumab levels as compared to those without an ARE. The majority of AREs were, however, preceded by changes in dosing of clinical immunosuppression and/or in relation to intercurrent (infectious) complications.

In SPKT, only a few retrospective and 2 small prospective studies have directly compared ATG with alemtuzumab induction therapy.^{16-19,23,24} Two retrospective studies reported, in contrast to the findings from our study, comparable numbers of AREs after ATG and alemtuzumab induction.^{16,19} In

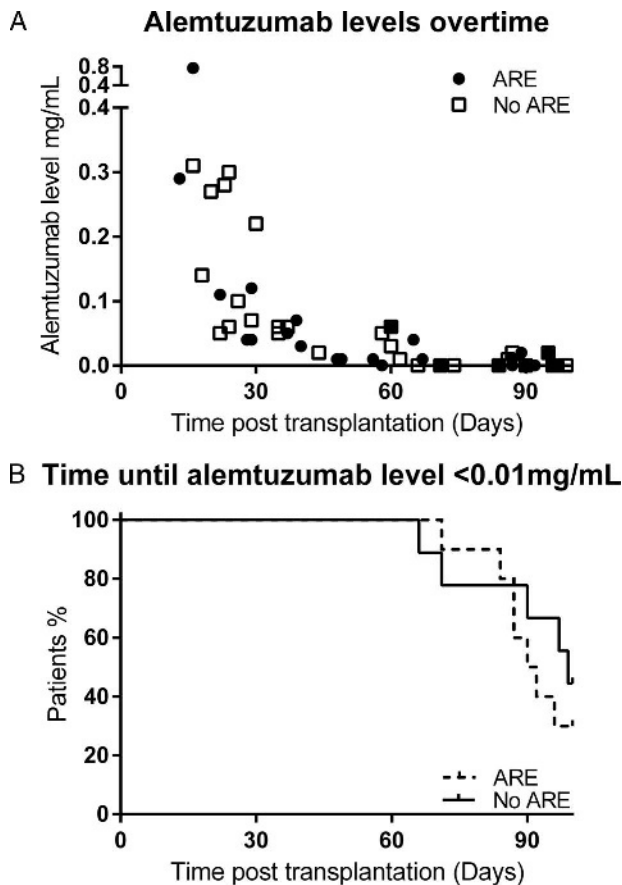


FIGURE 4. Late AREs were not associated with plasma alemtuzumab levels. A, Alemtuzumab levels posttransplant. In 13 of 20 patients (ARE $n = 7$, no ARE $n = 6$) alemtuzumab levels were no longer detectable at 90 days post transplantation. B, The number of days after transplantation until alemtuzumab levels were no longer detectable (<0.01 $\mu\text{g/mL}$) was analyzed. This was not different for patients with and without an ARE (log-rank 0.486).

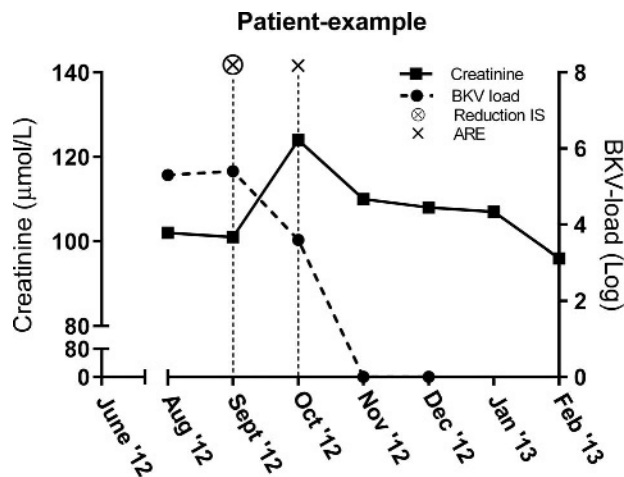


FIGURE 5. Patient example of empiric dose reduction and a subsequent ARE. Patient was transplanted in June 2012. In August 2012, patient suffered from a BKV and physician reduced MMF (500 mg twice daily) and TAC (1 mg twice daily). After 1 month with low-dose MMF and low TAC exposure (trough level, 3.6 µg/L) an ARE occurred. BKV staining in biopsy was negative. BK virus load (¹⁰ log of viral copy number/mL plasma).

both studies, maintenance immunosuppression posttransplantation was steroid-free, with TAC/MMF in 1 study,¹⁶ and TAC/sirolimus in the other.¹⁹ The prospective study by Stratta et al included 46 SPKT recipients who were treated with TAC/MMF maintenance, and steroids in case the patient was identified as high immunological risk.^{17,24} After 5 years of follow-up, number of AREs was comparable to the number found in our study (alemtuzumab [n = 6], 21%; ATG [n = 8], 44%; *P* = 0.11). Only 1 ARE was observed in the alemtuzumab group between 1 and 5 years posttransplantation. Detailed information about timing of the AREs within the first year was, however, not provided. The other prospective study consisted of 225 kidney transplant recipients, including 38 SPKT recipients, for whom the maintenance regimen was also based on immunological risk.^{18,23} Time to AREs was significantly longer in the alemtuzumab group (4.50 months) compared to ATG group (1.25 months); however, data regarding timing of AREs in SPKT recipients (n = 8) only was not reported.

Two prospective studies in renal transplantation also showed a delayed onset of AREs between 3 and 6 months.^{25,26} In both studies, patients received alemtuzumab induction followed by monotherapy TAC. Another prospective study in renal transplantation, with alemtuzumab induction and TAC/MMF maintenance, found higher incidence of AREs even later, between 12 and 36 months, posttransplantation.¹²

It has been postulated that repopulation of CD3⁺ T cells after alemtuzumab induction is associated with AREs later in time. Studies have shown that lymphocyte repopulation starts after 3 months, but may not return to baseline until 2 to 3 years posttransplantation.^{27,28} The exact repopulation kinetics for the current cohort could not be studied, due to the lack of consecutive sample collection.

In previous studies, an increase in regulatory B and T cells and a shift towards naive/transitional B cells has been described after alemtuzumab induction,^{27,29} which might create a tolerogenic environment. In this study we did, however, not observe a difference in regulatory B and T cells between patients with and without an ARE. It should be noted, however,

that only frozen blood samples were available and analyses were performed in retrospect, therefore the absolute number of T and B cells could not be determined.

Significant interpatient variability of plasma alemtuzumab levels have been reported from nonsolid organ transplant studies.^{30,31} In patients with chronic lymphocytic leukemia, higher alemtuzumab exposure resulted in a greater probability of a positive tumor response.^{30,31} Furthermore, patients with longer alemtuzumab exposure showed a delayed T cell and NK-cell recovery.³² It is, however, difficult to extrapolate the results from those studies to the field of organ transplantation, because the dose and frequency of alemtuzumab and the composition of lymphocytes at baseline differs. To our knowledge, in kidney transplant recipients only 1 study has investigated alemtuzumab plasma levels.³³ In this study, 30 mg alemtuzumab i.v. was given and plasma alemtuzumab levels were measured in 13 kidney transplant recipients early posttransplantation, up to 12 months. Levels declined from 1.3 µg/mL at 1 hour to less than 0.08 µg/mL at 1 month posttransplant and were no longer detectable beyond the first month. Since alemtuzumab was administered intravenously, it is difficult to compare these data with the results from the s.c. administration in our study. Levels determined in our study also showed high interpatient variability, ranging from 0.04 µg/mL to 0.3 µg/mL at 1 month, but could be detected up to 3 months posttransplantation. Of note, in our study we found no difference in alemtuzumab levels over time for patients with and those without an ARE. The impact and clinical relevance, however, of the observed interpatient variability in alemtuzumab levels needs to be investigated more thoroughly.

An important safety concern after alemtuzumab induction includes the degree of lymphocyte depletion and the number of subsequent (opportunistic) infectious complications. One prospective study in renal transplantation demonstrated that alemtuzumab and ATG had similar effects with respect to lymphocyte depletion and rate of recovery.¹² In contrast, several studies in renal and SPKT recipients, have reported more leukopenia following alemtuzumab induction, however, without more frequent or severe (opportunistic) infections.³⁴⁻³⁶ In this perspective, the dose of alemtuzumab may be of relevance. Higher CMV infection rates have been reported with an alemtuzumab dose of 60 mg i.v.,¹⁵ while studies with 30 mg found no differences in infectious complications.^{16-19,37} In the current study, alemtuzumab induction with 30 mg s.c. provided effective prophylaxis against early rejection, and was not associated with an increased risk of infection, also not on the long-term.

The finding that, in the alemtuzumab group, additional empiric dose reductions preceded the majority of late AREs may be of direct clinical importance. This may explain the apparent lack of differences in lymphocyte subsets and immune function between patients with and without AREs. By exclusion this suggests that adjustments in TAC and/or MMF dose, in the absence of steroids, might make SPKT recipients more susceptible for rejection. In the retrospective analysis of Muthusamy et al,³⁸ 25% of the pancreas transplant recipients suffered from an ARE after alemtuzumab and steroid-free maintenance regimen. Furthermore, 83% of the recipients remained steroid-free. They concluded that alemtuzumab induction can be carried out without CS maintenance. This was, however, not compared to a cohort who received maintenance with CS.

In kidney transplant recipients, a Cochrane review showed that AREs occurred more often after a steroid-free protocol.³⁹ The benefits of a steroid-avoidance protocol, including a reduction in lipids, new-onset diabetes, cataracts and the need of antihypertensive drugs, have not been evaluated in SPKT recipients. In our study, no differences in HbA1c, c-peptide and cholesterol levels were found, up to 3 years of follow-up. In line with this reasoning a recent Cochrane review in SPKT concluded that there is currently insufficient evidence to determine safety and efficacy of a steroid-free regime,⁴⁰ because large randomized controlled trials are lacking. In addition, the presumed metabolic benefits may be limited in patients with longstanding diabetes and established complications. Therefore, in the risk benefit equation of CS, their theoretical adverse metabolic effects should be carefully weighed against the risk of inadequate immunosuppression.

There are some obvious limitations inherent to the observational character of the current study. Adequately powered prospective trials in SPKT are, however, less feasible given the relative low number of annual transplants performed. There was a significant difference in the cold ischemia time between the ATG and alemtuzumab group (11.6 ± 2.5 vs 10.0 ± 2.9 hours, respectively); however, this did not result in a higher incidence of DGF. Furthermore, the number of patients with a preemptive transplantation and direct enteric drainage was significantly different. Although these above described differences related to the timing of transplantation, this was a relatively homogenous population of patients with type 1 diabetes, end-stage renal disease and a SPKT. When taking these issues into account in the Cox regression analysis, the incidence of ARE remained significant different for the ATG and alemtuzumab group. Unfortunately, we were unable to compare BKV infections between both groups, because prospective BKV monitoring was only routinely performed from 2007 onward.

In conclusion, in this cohort study of SPKT recipients, we have shown that alemtuzumab induction with a steroid-free regime was associated with a significant reduction in 3-year AREs as compared with ATG followed by triple maintenance therapy. Most AREs after alemtuzumab induction occurred beyond 3 months posttransplantation and were not associated with an altered subset distribution of the lymphocytes or kinetics of decline of plasma alemtuzumab levels. Late AREs were, however, frequently preceded by empiric dose reductions in TAC and/or MMF after intercurrent (viral) infectious complications. This observation suggests that these empiric dose adjustments, in the absence of steroids, constitute an increased risk for subsequent rejection in SPKT. Adding low-dose steroids to this regime may be a safer strategy to prevent late AREs in SPKT, while the presumed metabolic benefits versus low-dose steroids may be more redundant in patients with longstanding diabetes and established complications.

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