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Absence of Intragraft B Cells in Rejection Biopsies After Rituximab Induction Therapy: Consequences for Clinical Outcome

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Background. The pathophysiological role of intragraft B cells during renal allograft rejection is unclear. **Methods.** We studied B-cell infiltration during acute rejection in 53 patients who participated in a clinical trial in which adult renal transplant patients were randomized between a single intraoperative dose of rituximab (375 mg/m²) or placebo as induction therapy. Two independent pathologists scored all biopsies in a blinded fashion according to the Banff classification and scored for the presence of B cells and plasma cells using CD79a and CD138 as markers. **Results.** The majority of acute rejections were T cell-mediated. The proportion of acute rejections with an antibody-mediated component tended to be lower in rituximab-treated patients (4/23, 17.4%) than in placebo-treated patients (11/30, 36.7%; $P = 0.14$). Biopsies of rituximab-treated patients had significantly lower scores for B cells (0.00; range, 0.00-0.50 vs 1.70; range, 0.60-3.30; $P < 0.0001$) and plasma cells (0.10; range, 0.00-1.90 vs 0.40; range, 0.00-7.50; $P = 0.006$). During acute rejection, intragraft clusters of B cells were not observed after rituximab induction therapy. However, the depletion of intragraft B cells during acute rejection did not affect steroid resistance, proteinuria, graft function at 2 years follow-up, or patient and graft survival at a median follow-up of 4.1 years (range, 2.0-6.2 years). **Conclusions.** These data do not support a harmful influence of intragraft B cells present during acute allograft rejection on the clinical course within the first few years after renal transplantation.

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During acute allograft rejection, different cell types can infiltrate the graft, such as T cells, NK cells, monocytes, and also B cells. The clinical relevance of infiltrating B cells is a matter of debate. Some studies show an association with a poorer response to antirejection therapy and hence worse graft outcome, whereas other studies do not show a negative impact of B cell infiltration on graft outcome.¹⁻⁴

Rituximab is a monoclonal antibody against the CD20 antigen present on different types of B cells. After administration of a single dose, it rapidly induces complete and

long-lasting B cell depletion in the peripheral blood.⁵ However, in secondary lymphoid organs, like lymph nodes and spleen, B cell depletion after a single dose of rituximab is incomplete.^{6,7} Many case series have suggested a beneficial effect of rituximab in the treatment of (antibody-mediated) renal allograft rejection, but these results are difficult to interpret without a control group. In a small randomized trial ($n = 20$), treatment with rituximab (next to antithymocyte globulin and/or high-dose steroids) resulted in a larger improvement of graft function and of biopsy rejection scores

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at six months post-treatment, compared to treatment with antithymocyte globulin and/or high dose steroids alone.⁸ This improvement was accompanied by complete intra-graft B-cell depletion in all rituximab-treated patients at follow-up. Although rituximab had no apparent effect on donor specific antibody levels, reappearance of C4d deposition was not seen on follow-up biopsies after rituximab treatment. These findings suggest a pathogenic role of intra-graft B cells in acute renal allograft rejection.

In addition to its use for the treatment of rejection, rituximab has been studied for prevention of acute rejection. We have performed a double-blind, placebo-controlled study, in which 280 adult renal transplant patients were randomized between a single dose of rituximab (375 mg/m²) or placebo during transplant surgery.⁹ Patients were stratified according to panel-reactive antibody value and rank number of transplantation. Maintenance immunosuppression consisted of tacrolimus, mycophenolate mofetil, and steroids. This study showed that a single dose of rituximab as induction therapy did not reduce the overall incidence of biopsy proven acute rejection, but might be beneficial in patients with higher immunological risk. Other studies have shown similar results in immunologically low-risk patients, although one small study reported an increased risk of rejection with rituximab therapy.^{10,11}

Here we compare graft histology during acute rejection in patients with and without rituximab as induction therapy, with emphasis on the effect of rituximab induction therapy on type of rejection (T cell-mediated versus antibody-mediated) and intra-graft B cell numbers. The main research questions were whether rituximab induction treatment leads to depletion of intra-graft B cells during acute rejection and whether this depletion is associated with a favorable outcome (less steroid-resistance and improved graft function after follow-up).

MATERIALS AND METHODS

The full details of the original clinical trial have been described elsewhere.⁹ This trial was approved by the Committee on Human-Related Research Arnhem-Nijmegen, conducted according to the Declaration of Helsinki and good clinical practice guidelines. In brief, 280 patients who underwent a renal transplantation in the Radboud university medical center, Nijmegen, The Netherlands, were randomized to treatment with a single intra-operative dose of 375 mg/m² body surface area rituximab (n = 138) or placebo (n = 142) added to standard immunosuppression consisting of tacrolimus, mycophenolate mofetil, and steroids. In this trial, biopsies were only performed on clinical indication. First line treatment of acute rejections consisted of methylprednisolone, followed by anti-T cell antibodies (rabbit antithymocyte globulin [Thymoglobulin], Genzyme; Muromonab-CD3 [OKT3], Janssen-Cilag; alemtuzumab [Campath], Genzyme) in case of steroid-resistance, defined as lack of improvement of graft function within five days after the first methylprednisolone dose.

We selected all patients who had a biopsy proven acute rejection within 6 months posttransplantation. We excluded biopsies in which no rejection was diagnosed, because a cellular infiltrate was generally absent in these cases. To rule out an

effect of earlier antirejection treatment on graft histology, we only analyzed the first rejection biopsy in each patient.

The biopsy material was bouin-fixated and four-micrometer sections were processed for routine histologic stains including hematoxylin-eosin, Jones' silver stain, Masson Trichrome, and periodic acid-Schiff after diastase treatment. Staining for C4d was performed on frozen sections using immunofluorescence technique, with a mouse polyclonal antihuman C4d antibody (Biogenesis Inc., Ede, The Netherlands). Four-micrometer sections were incubated with monoclonal antibodies directed at the B cell marker CD79a (M7050, clone JCB117 by Dako, Glostrup, Denmark) and the plasma cell marker CD138 (ILM3825-c1-clone B-A38 by Immunologic, Klinipath, Duiven, The Netherlands). As secondary antibody we used powervision Poly-HRP-antimouse/rabbit/rat IgG (Immunologic, Klinipath, Duiven, The Netherlands). Detection was carried out with the use of peroxidase as label and diaminobenzidine as substrate. We did not stain for CD20, because that staining could be falsely negative due to blockage of CD20 by rituximab. Moreover, we also did not use CD19 as a B cell marker, because a monoclonal antibody against CD19 for use in bouin-fixated tissue is not available. Because CD79a is expressed on B cells as well as plasma cells we performed a simultaneous staining for CD138 to differentiate between B cells and plasma cells.

Two independent pathologists scored all biopsies in a blinded fashion. Rejection was scored according to the Banff 07 criteria for T cell-mediated rejection (TCMR) and antibody-mediated rejection (AMR).¹² According to the Banff 07 classification, diffuse C4d staining (ie, >50% of peritubular capillaries) was defined as positive. To differentiate between clusters and scattered positive cells, CD79a+ cells were scored in a manner as previously described (for CD20+ and CD3+ cells) and CD138+ cells were scored as the number of positive cells per high power field.⁴ Per biopsy specimen the whole cortical area was examined. A cluster of B cells was defined as more than 30 CD79a+ cells without the interposition of tubules. For each high-power field, the scattered CD79a+ cells were scored according to an ordinal scale ranging from 0 to 5, as defined in Table 1. For each biopsy specimen, the total scores for CD79a+ cells, and the total numbers of CD79a+ clusters were divided by the number of high-power fields that were examined.

Statistical testing was performed according to distribution and type of data (unpaired *t* test, Mann-Whitney U test, χ^2 test, or Fisher exact test). Allograft loss and death were analyzed with the Kaplan-Meier method, and differences were assessed by the log-rank test. Correlations between histological

TABLE 1.
Histological scoring of interstitial CD79a+ cells

Histological scoring	No. positive cells per high power field
0	0-2
1	3-10
2	11-20
3	21-40
4	41-80
5	>80

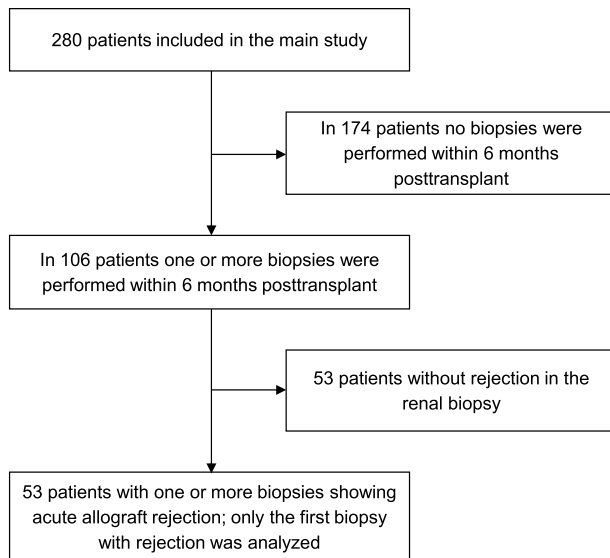


FIGURE 1. Trial profile of all patients.

scorings of CD79a and CD138 with clinical outcome were assessed with Spearman ρ correlation coefficient. Analyses were performed with IBM SPSS Advanced Statistics 21.0 and GraphPad Prism 5.03.

RESULTS

The clinical parent study was performed in 280 renal transplant patients (138 rituximab-treated vs. 142 placebo-

treated). Overall, the groups were well balanced with respect to recipient and donor characteristics.⁹ The number of patients who underwent at least 1 renal biopsy was 46 (33%) in the rituximab group and 60 (42%) in the placebo group ($P = 0.12$ by χ^2 test). An acute rejection was diagnosed in half of these patients (53/106), 23 rituximab-treated and 30 placebo-treated (Figure 1). The rituximab and placebo-treated patients with acute rejection were balanced with respect to demographic and clinical characteristics (Table 2). The median time from transplantation to rejection-biopsy was approximately 14 days (range, 2-180 days).

The results of the histological examination are shown in Table 3 and Figure 2. With respect to the type of rejection, AMR and AMR combined with TCMR were less frequent in rejection-biopsies from patients treated with rituximab, but this was not statistically significant (17.4% vs 36.7%, $p = 0.14$ by Fisher exact test). No differences between groups were observed in other rejection-related items of the Banff scheme such as tubulitis, (t score), arteritis (v score), inflammation (i score), and glomerulitis (g score) (Figure 2).

The score for interstitial CD79a + cells was significantly lower in rituximab-treated patients (median, 0.00; range, 0.00-0.50), compared with placebo-treated patients (median, 1.70; range, 0.60-3.30; $P < 0.0001$ by Mann-Whitney U test, Figure 3). Clusters of CD79a + cells were found in the biopsies of four placebo-treated patients while these were never present in biopsies of rituximab-treated patients. Interestingly, in placebo-treated patients the score for interstitial CD79a + cells was significantly lower in patients with

TABLE 2.
Baseline characteristics of patients with biopsy-proven acute rejection^a

Variable	Rituximab (n = 23)	Placebo (n = 30)
Age, y	47.3 ± 13.5	51.2 ± 11.4
Male sex (%)	56.5	53.3
White race (%) ^b	95.7	96.7
Cause of end-stage renal disease (no. patients)		
Glomerulonephritis	5	14
Diabetes mellitus	1	3
Urological disorder	5	4
Hypertension/vascular damage	2	0
Polycystic kidney disease	7	4
Uncertain or other	3	5
Type of donor (%)		
Living	56.5	53.3
Deceased donation after circulatory death	4.3	20.0
Deceased donation after brain death	39.1	26.7
Donor age (yr)	55.1 ± 8.6	52.3 ± 11.0
HLA mismatches—A, B, and DR (n)	3.26 ± 1.10	3.20 ± 1.30
Panel-reactive antibody titer—highest assessment	2 (0-83)	2 (0-71)
Patients with retransplant (%)	8.7	20.0
Cold-ischemia time—deceased donors only, h	17.0 ± 4.8	16.1 ± 4.1
Days between transplantation and rejection biopsy, d	14 (3-180)	13 (2-161)
Calculated GFR at time of rejection, mL/min ^c	19 (0-40)	17 (0-48)

^a Values are presented as mean ± standard deviation or median (range). Overall group differences were not significant.

^b Race was determined by the investigator.

^c For the calculated GFR on the basis of abbreviated Modification of Diet in Renal Disease criteria, the following formula was used: estimated GFR (mL/min per 1.73 m²) = 175 × (serum creatinine/88.4)^{-1.154} × (age)^{0.203} × (0.742 if female) × (1.212 if African American). In case of continued dialysis treatment, a GFR of 0 mL/min was used.¹³

TABLE 3.**Incidence and type of biopsy proven acute rejection at 6 months^a**

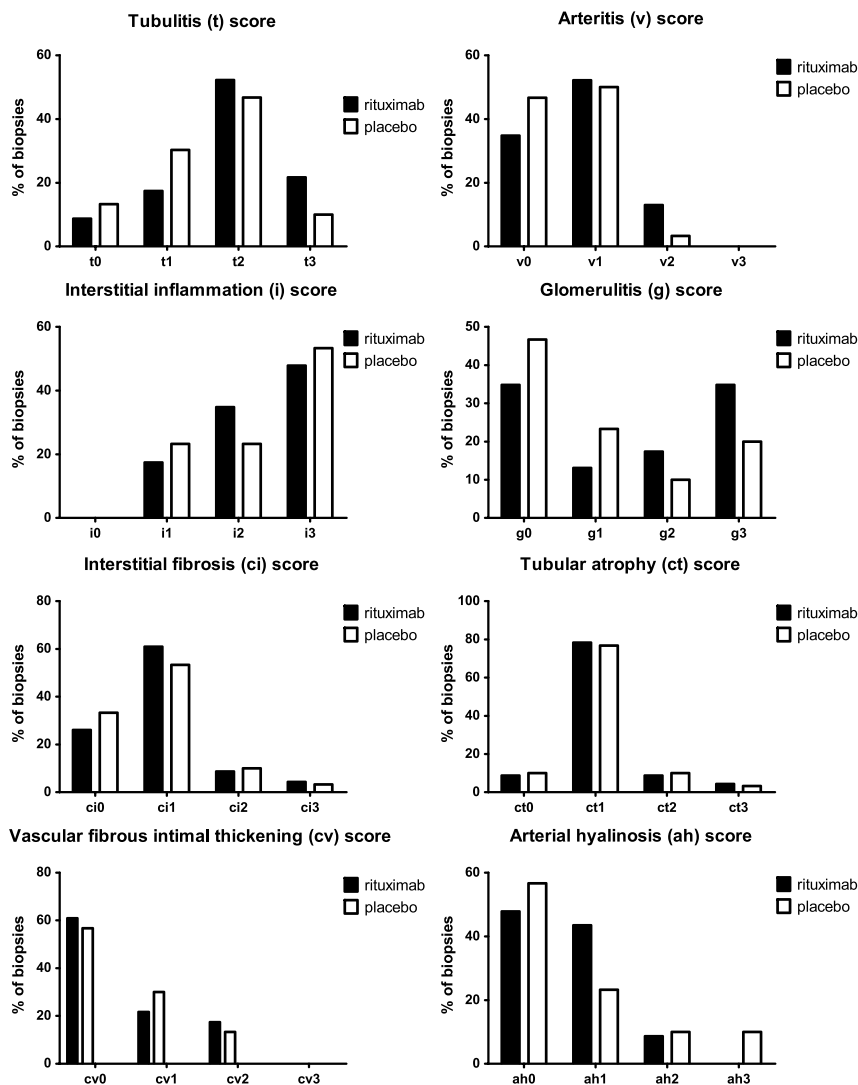
Variable	Rituximab (n = 23)	Placebo (n = 30)
TCMR (no.)		
Type IA	5	6
Type IB	2	2
Type IIA	10	9
Type IIB	2	2
AMR, n	1	2
Combined rejections (no.)		
AMR + Type IIA	2	9
AMR + Type IIB	1	0
C4d positive, n (%)	4 (17.4)	11 (36.7)

^a Biopsies were independently scored by 2 pathologists according to the Banff 07 classification.¹² A diagnosis of AMR required positive immunostaining for C4d, combined with either signs of microvascular inflammation ($g > 0$ and/or $ptc > 0$) or intimal arteritis ($v > 0$).

AMR (median, 1.00; range, 0.60-2.00) than in patients without AMR (median, 2.00; range, 1.00-3.30; $P = 0.002$ by Mann-Whitney U test).

The score for interstitial CD138 cells was also significantly lower in rituximab-treated patients (median, 0.10; range, 0.00-1.90) compared with placebo-treated patients (median, 0.4; range, 0.00-7.50; $P = 0.006$ by Mann-Whitney U test, Figure 3), although considerable overlap between the groups existed. Clusters of CD138+ cells were found in the biopsies of 2 placebo-treated patients, and not in biopsies of rituximab-treated patients. None of the biopsies showed evidence of the formation of ectopic lymphoid tissue (as in tertiary lymphoid organs).

Although induction with a single intraoperative dose of rituximab led to an almost complete absence of intra-graft CD79a+ cells at the time of rejection, this did not translate into a beneficial effect on the subsequent clinical outcome. The percentage of steroid-resistant rejections did not differ between rituximab and placebo-treated patients (43.5% vs 50.0%, $P = 0.66$ by Fisher exact test). Furthermore, there was no difference in proteinuria or graft function at 2 years posttransplant. Patient and graft survival at the end of follow-up were comparable in both groups (Table 4; and Figure S2, SDC, <http://links.lww.com/PRSGO/A393>). In the placebo-treated patients, the biopsy score for interstitial CD79a+ and CD138+ cells were not correlated with either

**FIGURE 2.** Histological scores of biopsies during acute allograft rejection.

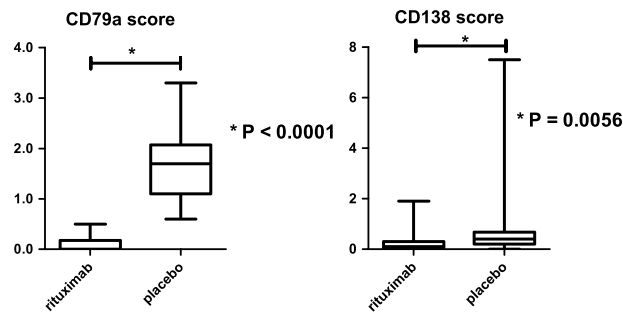


FIGURE 3. CD79a + and CD138+ scores in renal biopsies during acute allograft rejection.

improvement of estimated glomerular filtration rate (eGFR) (from moment of rejection to 24 months posttransplant) or absolute eGFR at 24 months posttransplant (**Figures S1a and S1b, SDC**, <http://links.lww.com/PRSGO/A393>). Of the four placebo-treated patients with CD79a + cell clusters in their biopsies, 3 had functioning grafts at the end of follow-up, while one patient died after 3 years with a functioning graft.

In 23 patients (12 in rituximab group; 11 in placebo group), antirejection therapy with methylprednisolone preceded the biopsy by at least 1 day. Because this could have influenced graft histology, we performed an additional analysis after exclusion of these 23 patients. In the remaining cohort of 30 patients (11 rituximab-treated and 19 placebo-treated patients) comparable results were found with respect to CD79a score (median score, 0.05; range, 0.00-0.50 vs 2.00; range, 0.70-3.30; $P < 0.001$) and again no correlation between B-cell depletion and clinical outcome was observed.

DISCUSSION

Infiltration of B cells is a frequent finding in biopsies of patients with acute renal allograft rejection. Our data show that in renal transplant patients who received a single intra-operative dose of rituximab as induction therapy, intra-graft B cells were nearly absent during subsequent episodes of acute allograft rejection. Moreover, the relative frequency of pure AMR or combined AMR and TCMR was lower than in placebo-treated patients. No differences were seen in the severity of tubulitis, arteritis, or the extent of the cellular infiltrate. Notably, the absence of intra-graft B cells during acute

rejection did not appear to affect the subsequent clinical course.

Like others, we previously showed that a single dose of rituximab at the time of renal transplant surgery results in a rapid and long lasting depletion of B cells in the peripheral blood.^{5,14} Even at 2 years after transplantation, the absolute number of B cells in peripheral blood was still quite low as compared with patients not treated with rituximab.¹⁴ We also showed that despite complete depletion of B cells in the peripheral blood the number of B cells in secondary lymphoid organs remained unaffected.⁶ Others have confirmed that in the near absence of B cells in peripheral blood there is a varying degree of reduction, but no complete depletion, of B cells in spleen, lymph nodes, and synovial tissue after treatment with rituximab.^{5,15-17} Together with our current findings, these data suggest that rituximab either inhibits the egress of B cells from secondary lymphoid organs or selectively depletes B cells in peripheral blood. In both cases, there is reduced migration of B cells to peripheral tissues such as the renal allograft.

Earlier studies by Hippen et al¹ and Sarwal et al,¹⁸ demonstrated that the presence of CD20+ B cell infiltrates in the graft at time of rejection is a bad prognostic sign. It was suggested that the antigen presenting function of infiltrating B cells might contribute to an augmented alloreactive response. Based on these findings, it could be expected that the absence of intra-graft B cells in rituximab-treated patients had resulted in a lower rate of steroid-resistance and better graft survival. However, our data do not indicate that absence of intra-graft B cells during acute rejection, as observed in rituximab-treated patients, translates into better outcome. The interpretation of this finding could be biased by a stronger severity of rejections occurring after B cell depletion by rituximab. This was unlikely however, because Banff scores for tubulitis, arteritis, inflammation, and glomerulitis were similar in biopsies of rituximab-treated and placebo-treated patients. Moreover, in the placebo-treated patients there was no correlation between the scattered or clustered presence of B cells and the clinical course. In four placebo-treated patients B cell clusters were found in the rejection biopsy, and after a median follow-up of 4 years 3 of the four patients still had a good and stable graft function. Notably, other authors were also unable to demonstrate that intra-graft B cells were associated

TABLE 4.

Outcomes after transplantation in patients with biopsy-proven acute rejection^a

Variable	Rituximab (n = 23)	Placebo (n = 30)
Patients with steroid-resistant rejection, n (%)	10 (43.5)	15 (50.0)
Calculated GFR at 2 y in patients with a functioning graft, mL/min ^b	33 (20-49)	38 (19-64)
Improvement of GFR from time of rejection till 2 y posttransplant in patients with a functioning graft at 2 y, mL/min	11 (-3 to 35)	18 (-5 to 55)
Proteinuria at 2 y, g/10 mmol creatinine	0.20 (0.1-0.8)	0.10 (0.1-4.0)
Allograft survival at end of follow-up (%) ^c		
Censored for death of patients with functioning graft	78.3	83.3
Uncensored for death of patients with functioning graft	60.9	70.0
Patient survival at end of follow-up (%) ^c	78.3	83.3

^a Values are presented as mean ± standard deviation or median (range).

^b For the calculated GFR on the basis of abbreviated Modification of Diet in Renal Disease criteria, the following formula was used: estimated GFR (mL/min per 1.73 m²) = 175 × (serum creatinine/88.4)^{-1.154} × (Age)^{-0.203} × (0.742 if female) × (1.212 if African American).¹³

^c median duration of follow-up, 4.1 years; range, 2.0-6.2 years.

with a poorer response to high dose steroids or worse graft survival.^{3,4,19-21}

As for the B cells, the number of intra-raft CD138+ plasma cells was lower in rituximab-treated patients than in placebo-treated patients. Although the clinical significance of this finding is uncertain, it suggests that intra-raft B cells can develop into plasma cells.

In the clinical trial overarching this study, a trend towards less AMR was seen in rituximab-treated patients, compared to placebo-treated patients (4/138, 2.9% vs. 11/142, 7.7% $P = 0.11$ by Fisher's exact test). Remarkably, placebo-treated patients with AMR had lower scores for interstitial CD79a+ cells than placebo-treated patients without AMR. Taken together, this suggests that any B cells and plasma cells that are involved in the process of AMR are residing outside the graft.

The interpretation of our data is somewhat limited by the moderate number of patients, and relatively short follow-up period. However, most rejections occurred within the first 3 months after transplantation and all patients had a follow-up of at least 2 years.

To conclude, induction therapy with rituximab strongly reduced the number of infiltrating B cells at the time of acute renal allograft rejection. This did not have an effect on the severity of tubulitis, arteritis or the extent of the cellular infiltrate, nor did it improve clinical outcome after treatment of the rejection. These data do not support previous findings suggesting that intra-raft B cells that are present during acute allograft rejection are harmful for the graft during a median follow-up of 4 years.

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