Tacrolimus versus cyclosporine induction therapy in pulmonary transplantation in miniature swine

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Abstract

Objective: Tacrolimus has been shown to provide superior immunosuppression in various solid organ transplant settings. The purpose of our study was to compare the survival of porcine lung allografts after induction with either cyclosporine A (CsA) or tacrolimus. Methods: Single lung transplantation from MHC mismatched donors was performed in 10 minipigs. Immunosuppression included 1.5 mg/kg per day methylprednisolone and 1.0 mg/kg per day azathioprine. CsA (n=5) was adjusted to trough levels of 300–500 ng/ml, tacrolimus (n=5) was adjusted to 16–26 ng/ml. All immunosuppressive drugs were discontinued on postoperative day (POD) 28. Allograft survival was monitored by sequential chest radiographs, bronchoscopy and transbronchial biopsy histology. Peripheral blood leukocytes were scanned for donor chimerism and CD3, CD4, CD8 and CD25 expression. Results: The animals survived a 4-week course of immunosuppression without radiological or histological signs of rejection on POD 28. Median allograft survival in CsA-treated animals was 55±15 days and all animals rejected their grafts within 42 days after withdrawal of immunosuppression. In tacrolimus-treated animals, median survival was 152±65 days with the longest survivor being electively sacrificed on POD 390 (P=0.0064). The degree of donor leukocyte chimerism and the frequency of CD4+CD25+T-cells were higher in the tacrolimus group, however, these differences were not statistically significant. Conclusion: The results of our study show that primary immunosuppression with tacrolimus is superior to cyclosporine after pulmonary allotransplantation in a large animal model.

Keywords: Lung transplantation; Tacrolimus; Cyclosporine; Primary immunosuppression

1. Introduction

Lung transplantation is an accepted therapeutic option for end-stage pulmonary disease. However, pharmacologic immunosuppression remains unsatisfactory with a high incidence of infections [1], frequent acute rejection episodes [2] and early onset of bronchiolitis obliterans [3,4]. Despite the introduction of new leukocyte-depleting antibodies and the inclusion of rapamycin into immunosuppressive protocols, the basis of currently applied protocols remains a calcineurin-inhibitor, i.e. cyclosporine or tacrolimus [5].

Tacrolimus has been shown to have a greater immunosuppressive potency than cyclosporine in various experimental settings [6–8]. However, the superiority of tacrolimus over cyclosporine as a primary or maintenance agent has not been established to date in clinical lung transplantation [5]. Several smaller single center studies could not show a significant survival benefit, but a reduction in the incidence of bronchiolitis obliterans for tacrolimus-treated patients [9–11]. While patients with recurrent acute or chronic lung allograft rejection are routinely switched from cyclosporine to tacrolimus in an often successful attempt to improve immunosuppression [12], the beneficial effect of primary administration of tacrolimus is less clear. Many centers therefore continue to use cyclosporine as the primary immunosuppressive agent after lung transplantation. This is at variance from experimental data, that has even shown the induction of long-term tolerance after a short course of tacrolimus in a fully MHC-mismatched porcine model of kidney transplantation [6].

Our study was designed to evaluate the effect of a primary 28-day course of tacrolimus as compared to cyclosporine on graft survival in a large animal model of pulmonary transplantation across major histocompatibility barriers.

2. Methods

2.1. Animals

All animals received humane care in compliance with the German animal protection legislation, the "Principles of
Laboratory Animal Care”, and the 'Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996.

From an outbred minipig herd, consisting of eight different breeding lines (Ellegaard, Dalmoose, Denmark), 20 animals between 12 and 15 months of age were selected. The animals were bred and kept in a specific pathogen-free (SPF-) facility. Donors and recipients were selected from different breeding lines, and prospectively tissue-typed by a lymphocytotoxic assay. Subsequently, they were mismatched for the swine leukocyte antigen (SLA) class I DC45, W12 or FJ13,W9 haplotypes [13,14] and for staining with the mAb 74-11-10 SLA I (haplotype d). Mixed lymphocyte reaction (MLR) was performed to test in vitro anti-donor reactivity before transplantation.

2.2. Surgical technique

Donor lungs were harvested from mechanically ventilated donors (18–25 kg) after Euro-Collins cold flush perfusion. A permanent vascular access double-lumen 3.2 mm Quinton catheter was inserted into the jugular vein of recipient animals (16–25 kg). After left-sided thoracotomy in the fourth intercostal space the lung was removed. The allogeneic lung was then transplanted using a telescoping bronchial anastomosis technique with running posterior wall and interrupted anterior wall 4-0 polydioxanone sutures. The atrial cuff and the pulmonary artery were anastomosed with running polypropylene sutures. The animals were extubated immediately after the procedure was finished. The animals received empiric intravenous antibiotic therapy with 200 mg Ciprofloxacin (Bayer, Ludwigshafen, Germany), divided into two daily doses.

2.3. Experimental groups and immunosuppression

Tacrolimus group recipients (n=5) received triple intravenous immunosuppression including 1.5 mg/kg per day methylprednisolone, 1.0 mg/kg per day azathioprine (Glaxo Wellcome, Middlesex, GB), both administered once daily, and tacrolimus (Fujisawa, Osaka, Japan), administered twice daily. The tacrolimus dose was adjusted daily in order to reach whole blood trough levels of 16-26 ng/ml.

Cyclosporine A group recipients (n=5) received triple intravenous immunosuppression including 1.5 mg/kg per day methylprednisolone, 1.0 mg/kg per day azathioprine, both administered once daily, and cyclosporine A (Novartis, Basel, Switzerland). The cyclosporine dose was adjusted daily in order to reach trough levels of 300-500 ng/ml.

Tacrolimus and cyclosporine A trough levels were monitored using standard radioimmunoassays. All immuno-suppressive medication and intravenous antibiotics were withdrawn on POD 28 and the vascular access catheter was surgically removed.

2.4. Rejection monitoring

Sequential chest radiographs were performed on post-operative days (POD) 7, 28, 42, 56, 72 and thereafter depending on the animal’s clinical course. The degree of radiologic lung infiltration was quantified by applying a score from 0 (no pathologic changes) to 4 (homogenous infiltration of the left lung, normal right lung) by a blinded reviewer (J.N.). On the same POD’s a fiberoptic bronchoscopy was performed. A broncho-alveolar lavage was performed in the lingula of the transplanted lung for microbiologic assessment and 3-6 transbronchial biopsies were taken from the left lower lobe. Paraffin-embedded sections of 5 μm thickness were stained with hematoxylin and eosin and reviewed by a blinded pathologist (P.F.). Histological acute and chronic rejection were graded referring to the ISHLT guidelines ranging from A0-A4 and from B0-B4, respectively [15]. Once the chest radiograph demonstrated a strictly left-sided infiltrate scored 3-4 and histology revealed a grade A2 to A4 or B3-B4 rejection while infection has been excluded, the animals were sacrificed by a thiopental overdose. A full autopsy was performed.

2.5. Isolation of PBMC and flow cytometric analysis

PBMC were isolated from heparinized whole blood or from lymphoid cell suspensions from spleen tissue by gradient centrifugation using lymphocyte separation medium (Ficoll-Paque, Amersham Pharmacia Biotech AB, Uppsala, Sweden). Mononuclear cells were washed twice in PBS and viable cells were counted after counterstaining dead cells with trypan blue. PBMC were adjusted to 1 × 10⁶/ml and distributed into tubes. Unspecific binding sites were blocked with normal porcine serum. The following mAb were used as primary antibodies: negative control mouse IgG2a, mouse IgG2b and mouse IgM (Beckman Coulter, Fullerton, CA, USA), CD3a BB23-B6 IgG1 (BD Pharmingen, San Diego, CA, USA), CD25 PBGL25A IgG1 (VMRD, Pullman, WA, USA), CD4 74-12-4 Balb/c IgG2bK, CD8 76-2-11 Balb/c IgG2aK, SLA class I 74-11-10 mlgG2b (harvested from cultured hybridoma cell supernatant, ATCC, Manassas, VA, USA). Flow cytometry was performed as a two-color-analysis. The SLA class I haplotype d mAb 74-11-10 was used to detect donor cell chimerism. Donor animals had prospectively been selected 74-11-10 positive, and recipient animals had been selected negative. The cells were stained with titrated concentrations of primary mouse-anti-pig antibody for 30 min at 4 °C, washed in PBS and visualized by the secondary phycoerythrin (PE) conjugated rat-anti-mouse IgG1 antibody (staining for 30 min at 4 °C). After washing the second primary mouse-anti-pig antibody was incubated for 30 min at 4 °C, stained and then stained with the respective secondary fluorescein isothiocyanate (FITC) conjugated rat-anti-mouse IgG2a or IgG2b Ab. After washing, the cells were analyzed on a FACScalibur flow cytometer (BD, San Jose, CA, USA). Before acquisition, propidium iodide was added to each tube to exclude dead cells. Data were analyzed using the BD Cell Quest and WinMdI 2.8 analysis software.

2.6. Mixed lymphocyte reaction

In vitro anti-donor reactivity of host lymphocytes was determined using a one-way-MLR. Isolated PBMC were adjusted to 1 × 10⁵ cells/ml. Donor (stimulator-) PBMC were irradiated at 20 Gy using a γ-irradiation source to inhibit proliferation. The cells were incubated in
flat-bottomed 96-well plates (Greiner, Germany) in culture medium consisting of RPMI 1640 (Gibco BRL, Gaithersburg, MD, USA) supplemented with $5 \times 10^{-5}$ M β-2 mercaptoethanol, 15% normal porcine serum, 20 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 135 U/ml streptomycin and $50 \mu g/ml$ gentamycin (all purchased from Sigma, St Louis, MO, USA). Each well contained $5 \times 10^4$ responder (recipient) and $5 \times 10^4$ irradiated stimulator PBMC. Responder cells were incubated with their respective donor’s stimulator cells, with PHA (phycohemagglutinin) as a positive control or with medium alone as a negative control. Each combination was run in quadruplicate. Cells were cultured for 5 days at $37^\circ C$ and 100% humidity. For the final 18 h of culture, plates were pulsed with $^3H$-thymidine. $^3H$-thymidine uptake was measured in a β-counter, and results were expressed as a stimulation index (SI):

$$SI = \frac{\text{Experimental cpm} - \text{Antimedium cpm}}{\text{Antimedium cpm}} \times 100 \%$$

(cpm: counts per minute)

2.7. Statistical analysis

Graft survival was compared between groups using Kaplan-Meier-survival curves. Spearman’s correlation coefficients for nonparametric data were calculated to show or rule out influences of cumulative drug levels (areas under the curve) on graft survival using two-tailed testing. $P$ values of less than 0.05 were considered significant. Repeated-measures analysis of variance (ANOVA) was used to compare data with repetitive measurements.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Summary of animals</th>
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<tr>
<td>Animal No.: recipient-donor</td>
<td>POD of death/survival</td>
</tr>
<tr>
<td>CsA group</td>
<td></td>
</tr>
<tr>
<td>80441-60179</td>
<td>55</td>
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<tr>
<td>60178-60148</td>
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<tr>
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<td>83</td>
</tr>
<tr>
<td>64608-65117</td>
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</table>

3. Results

3.1. MHC mismatch

Typing of animals with the available haplotype-specific antisera for SLA class I occurring in our minipig breed led to detection of 75% of haplotypes. Thus mismatch could be ascertained in some donor-recipient pairs for two SLA class I haplotypes, but only for one haplotype in other pairs (Table 1). Stimulation indices from pretransplantation MLR show low to medium baseline alloreactivity, suggesting MHC disparity, but not indicating presensitization or the occurrence of cross-reactive T-cell memory (Table 2).

3.2. Pulmonary allograft survival

Pulmonary allograft survival is summarized in Table 1 and survival curves are given in Fig. 1. Transient acute rejection occurred in four out of five animals from the CsA-treated group but only in two out of five tacrolimus-treated animals, as determined by protocol chest radiographs (not shown) and bronchoscopic evaluation. At the day of immunosuppressive drug withdrawal, four CsA and five tacrolimus-treated animals revealed normal postoperative findings in bronchoscopy and chest radiography without signs of rejection.

![Fig. 1. Cumulative survival of lung allografts. *Statistically significant difference between the two experimental groups (Kaplan-Meier survival curves).](image-url)
Fig. 2. Chest radiographs performed on the day before withdrawal of pharmacologic immunosuppression (POD 27) or before sacrifice of the animal are given in the left or right columns, respectively. In the CsA group, animals 80441, 60709 and 62748 revealed normal postoperative radiologic findings on POD 27 but developed severe infiltration of the left lung consistent with rejection on POD 89, 69 and 69, respectively. Animal 60178 already showed infiltration of the left lung graded A3 on POD 27 that increased to A4 on POD 33. In animal 60642, a basal pneumothorax with partial lower lobe atelectasis is evident on POD 27, which was in part resolved on POD 41, but transparency of the upper aspect of the left hemithorax was decreased by POD 41, indicating rejection. In all tacrolimus-treated animals, the chest radiograph taken on POD 27 showed normal postoperative findings. Left-sided radiologic infiltration indicative of rejection was least pronounced in animal 85344, that showed a remarkably stable course for more than a year after transplantation.

All animals retained their preoperative weight. Animal 60178 (CsA) showed a subtotal bronchial stenosis due to proliferation of the graft mucosa on POD 27 and the respective loss of ventilation of the left lung in the chest radiograph (Fig. 2). While the left main bronchus could be reopened by bronchoscopic intervention, follow-up bronchoscopy showed recurrent obstruction on POD 33, with the corresponding chest radiograph shown in Fig. 2. The left lung biopsy taken at sacrifice on POD 36 showed grade 2 rejection. The remaining four CsA-treated animals developed coughing and severe infiltration of the left lung in the chest radiograph at various timepoints consistent with acute rejection and were accordingly sacrificed. Histology revealed perivascular mononuclear infiltrates in the left lungs graded A2-A4 (Table 1, Fig. 3). Median allograft survival in CsA animals was 55 ± 15 days. In the tacrolimus group, only two animals showed comparable early rejection (Nos. 65952, POD 83 and 83913, POD 90). Animals 64608, 65945 and 85344 had normal chest radiographs well above 100 postoperative days (not shown). Transbronchial biopsies (Fig. 3) and bronchoalveolar lavage fluid differential cell counts (data not shown) showed normal findings at various timepoints beyond 100 postoperative days in these three animals. Animal 64608 developed late progressive infiltration of the left lung and autopsy on POD 152 revealed acute grade 4 rejection. Radiological opacity of the left lung developed much slower in the remaining animals 65945 and 85344, and as opposed to acutely rejecting animals, there was no marked increase in neutrophil counts in bronchoalveolar lavage fluid.

### 3.3. Drug whole blood trough levels

Cyclosporine trough levels of 300-500 ng/ml and tacrolimus trough levels of 16-26 ng/ml were satisfactorily achieved throughout the experiment (Fig. 4). Maintenance dose was around 10 mg/kg CsA twice daily or 0.05 mg/kg tacrolimus twice daily. The area under the curve of CsA or tacrolimus blood levels of individual animals was not correlated with allograft survival (CsA: Spearman’s
correlation coefficient $-0.80$, $P = 0.104$; tacrolimus: Spearman’s correlation coefficient $-0.10$, $P = 0.862$). Serum creatinine levels remained stable without any differences to preoperative levels in all animals (data not shown).

3.4. Analyses of leukocytes from peripheral blood

Total leukocyte counts peaked around POD 8 in CsA animals, indicative of acute rejection (Fig. 5). In tacrolimus-treated animals, leukocyte counts were high early after reperfusion and steadily decreased thereafter without evidence of acute rejection (Fig. 5). Differences between groups were statistically not significant ($P = 0.291$, repeated-measures ANOVA). Lymphocyte counts were lower in the tacrolimus group (Fig. 5). However, that difference did not reach statistical significance ($P = 0.055$, repeated-measures ANOVA). In the CsA group, FACS analysis from PBMC revealed a decrease of CD4$^+$ lymphocytes during the observation period (before transplantation, 30.4$\pm$5.6% CD4$^+$ in the lymphocyte gate; POD 28 and thereafter, 19.8$\pm$6.1% CD4$^+$ in the lymphocyte gate). The respective CD8$^+$ counts increased in the CsA group (before transplantation, 35.4$\pm$7.6% CD8$^+$ in the lymphocyte gate; POD 28 and thereafter, 49.0$\pm$8.1% CD8$^+$ in the lymphocyte gate). Before transplantation 12.7$\pm$5.2% of the CD4$^+$ T-cells were of the CD4$^+$CD25$^+$ phenotype and that percentage increased to 18.9$\pm$11.45% after withdrawal of immunosuppression (Fig. 6A). In the tacrolimus group, neither a decrease of CD4$^+$ nor a significant increase in CD8$^+$ T-cells could be seen after transplantation. In tacrolimus-treated animals, 9.4$\pm$4.52% of the CD4$^+$ T-cells before transplantation and 18.3$\pm$14.73% after withdrawal of immunosuppression were of the CD4$^+$CD25$^+$ phenotype. Differences between groups with respect to CD4$^+$CD25$^+$ T-cell percentages or absolute counts were statistically not significant.

3.5. Peripheral blood chimerism

Chimerism detected by the donor-specific SLA class I mAb 74-11-10 peaked within 24 h of pulmonary transplantation (samples were drawn 1 h after reperfusion) and decreased continuously thereafter (Fig. 6B). Donor cells of the monocyte/macrophage and lymphocyte lines in the range of 0.5-2% were detectable in some animals for more than 80 days post transplant. Donor cell chimerism tended to be higher in tacrolimus-treated animals, although the difference between groups was not statistically significant. There was no significant correlation between the degree of peripheral blood leukocyte chimerism and allograft survival.

3.6. Mixed lymphocyte reaction

Anti-donor activity of recipient PBMC in the MLR was present at baseline. Anti-donor stimulation indices decreased under immunosuppression in the CsA group, but transiently increased in the tacrolimus group. In the event of rejection, stimulation indices increased more in the tacrolimus than in the CsA group (Table 2).
4. Discussion

The early immunologic response to an allograft has an important impact on its ultimate fate [16-18]. The frequency of early acute rejection episodes after lung transplantation has been firmly linked to the eventual development of bronchiolitis obliterans, i.e. chronic rejection [3,4,19]. Therefore, it may be of paramount importance to control early alloreactivity by means of efficient but safe immunosuppression to improve long-term results after lung transplantation. Prevention of acute rejection relies in the first instance on steroids and the calcineurin inhibitors cyclosporine A and tacrolimus [5]. While the steroid dose is aimed at early tapering to reduce adverse effects, early variations of calcineurin inhibitor blood levels might have profound effects. Cyclosporine blood trough levels in excess of 300 ng/ml and tacrolimus trough levels above 16 ng/ml are considered prohibitive in humans, primes and dogs due to nephrotoxicity [20]. Mice, rats and pigs tolerate comparably excessive levels of calcineurin inhibitors without any adverse effects [20] and therefore studies in these species have to be carefully interpreted with regard to comparability with humans.

A major difficulty in setting up the experiments for this study was defining reasonable target levels of CsA and tacrolimus that should resemble equivalent effective doses in humans. Pigs have been shown to require higher CsA doses than humans to achieve equivalent blood levels [21]. The time course of immunosuppressive therapy was restricted to only 28 days in our study and we therefore aimed at drug blood levels slightly higher than the clinical target level, but within the same equivalent blood levels used in humans. Thus, 20 ng/ml CsA is approximately equivalent to 1 ng/ml tacrolimus, leading to administered doses of ca. 10 mg/kg CsA or 0.05 mg/kg tacrolimus twice daily intravenously in our experiments. This is opposed to data from a study in rats that used daily oral administration of 10 mg/kg CsA and 3.2 mg/kg tacrolimus as equivalent effective doses as determined by comparison of rat heart allograft survival [7]. Differences in drug metabolism that selectively affect tacrolimus bioavailability and pharmacokinetics or its potency as compared to large animals and humans may therefore prevail in rodents. In addition, cytochrome P450 gene polymorphisms [22], variations in food intake or internal absorption [23] as well as drug interactions might considerably influence calcineurin inhibitor blood levels not only in humans, but also in experimental animals. To maintain reliable blood levels and consistent experimental data, it is therefore crucial to adjust the, preferably intravenously, administered dosed to measured levels.

Primary immunosuppression with tacrolimus resulted in significantly prolonged porcine lung allograft survival when compared to CsA-treated animals in our study. This finding supports evidence for superior immunosuppression provided by tacrolimus in lung transplantation. While in our study mild transient acute rejection was found on POD 7 in most animals from the CsA group, this was only found in two tacrolimus-treated animals. After withdrawal of immunosuppressive medication, acute rejection occurred within weeks in CsA animals, but was markedly delayed or even abrogated in tacrolimus animals. However, tolerance was not induced, since all tacrolimus animals eventually developed changes of the graft consistent with either acute or chronic rejection. On the contrary, Utsugi [6] described the induction of tolerance to fully MHC-mismatched kidney allografts using a short course of tacrolimus in a porcine model. That study differed from our experiments mainly in the higher tacrolimus blood trough levels of 45-80 ng/ml in animals that eventually accepted their grafts long term. The investigators defined a threshold blood level for tolerance induction of approximately 35 ng/ml tacrolimus within a short postoperative course of drug administration. Although the authors of that study claim clinical relevance for their protocol, to our knowledge it has not been applied in clinical transplantation to date, presumably because major toxicity at such high tacrolimus levels is anticipated. All our experimental animals had blood levels below that threshold, in line with the finding that tolerance was not seen in our study.

Analysis of circulating leukocytes revealed an early decrease of CD4+ and increase of CD8+ T-cells in CsA but not in tacrolimus animals in our experiments. A subset of the CD4+CD25+ T-cells has been reported to elicit regulatory function and thereby suppresses anti-donor effector T-cells in various animal models and potentially in humans [24]. Flow cytometry was used to investigate, if the relatively higher peripheral blood CD4+ counts in tacrolimus animals would translate into an increased occurrence of potentially regulating CD4+CD25+ T-cells. The percentage of T-cells with a CD4+CD25+ phenotype increased without differences in both groups during the observation period, although total numbers were higher in the tacrolimus group. The higher total numbers of potential regulatory T-cells in tacrolimus-treated animals might explain prolonged graft survival. However, the relative increase of T-cells of the CD4+CD25+ phenotype in all experimental animals could merely indicate an increased occurrence of antigen experienced CD4+ T-cells. Further studies are necessary to elucidate functional properties of regulatory T-cells in our model, since differences in suppressive function might not necessarily be reflected by differences in cell numbers. Maintained alloreactivity was found in MLR assays in our experiments, irrespective of the presence of increased percentages of CD4+CD25+ T-cells. MLR stimulation indices were even higher in the tacrolimus group. At no time after transplantation was in vitro anti-donor activity completely abrogated as would have been expected in tolerant recipients. Donor leukocyte chimerism could be detected in all experimental animals at varying degrees in peripheral blood early after transplantation. The tendency of an initially more pronounced donor cell chimerism in tacrolimus animals might be explained by a more efficient functional inhibition of recipient alloreactive T-cells in the tacrolimus group, thus circulating donor cells are cleared slower than in CsA animals.

4.1. Limitations of the study

The incompleteness of the SLA typing in our experimental animals, especially with respect to MHC class II disparity is a limitation of our animal model. However, while a partial MHC-match cannot be ruled out, it is unlikely, that
the observed differences between the groups can be explained solely on grounds of random SLA-matches. In our study groups, approximately 75% of the SLA class I haplotypes could be typed, low but consistent MLR reactivity was shown in all donor-recipient pairs and all animals eventually rejected. While incomplete MHC typing is a common problem in outbred large animal models [25], those based on the use of inbred strains exclude the heterogeneity that prevails in human populations.

We conclude, that primary immunosuppression with tacrolimus for 28 days leads to significantly prolonged pulmonary allograft survival compared to initial therapy with cyclosporine in our large animal model. This prolongation of survival was associated with a reduced incidence and delayed onset of acute rejection. Transplantation tolerance was neither observed in cyclosporine, nor in tacrolimus-treated animals.

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