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Efficacies of sirolimus (rapamycin) and cyclosporine in allograft vascular disease in non-human primates: trough levels of sirolimus correlate with inhibition of progression of arterial intimal thickening

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Abstract We investigated the efficacies of sirolimus (rapamycin) and cyclosporine for inhibition of graft vascular disease (GVD) in cynomolgus monkey recipients of aortic allografts. Increases in arterial intimal thickening in the midgraft (six consecutive cross-sections) after transplantation were quantified by serial intravascular ultrasound (IVUS) from day 21 to day 105. These data enabled correlations between changes in intimal indexes [II = (intimal area/vessel area) × 100] and trough levels of sirolimus and cyclosporine to be determined. Eighteen recipients received no immunosuppression for 6 weeks to allow alloimmune injury to occur. On day 45, monkeys were treated daily with sirolimus ($n = 6$) or cyclosporine ($n = 6$); six monkeys remained untreated. II increased significantly from day 63 to day 105 in untreated monkeys and monkeys treated with cyclosporine, whereas monkeys treated with sirolimus did not have a significant increase in II ($P = 0.008$, $P = 0.006$, P = NS; paired t-test). The change in II from days 63 to 105 was significantly greater in untreated monkeys compared to sirolimus-treated monkeys ($P = 0.13$; one-way ANOVA, $P = 0.012$ Tukey's post hoc test); other post hoc pairwise comparisons were not significant. Mean sirolimus and cyclosporine levels ± SEM were 43 ± 7 ng/ml and 562 ± 20 ng/ml, respectively. Sirolimus trough levels, but not cyclosporine levels, correlated inversely with changes in II from day 42 to 105 ($r^2 = 0.73$, $P = 0.03$). This non-human primate study shows that inhibition of intimal thickening by sirolimus depends on trough levels and provides the rationale for clinical trials of sirolimus for the control of GVD in organ transplant recipients.

Key words Drug levels · Chronic rejection · Non-human primate · Intravascular ultrasound · Cyclosporine · Sirolimus (rapamycin)

Introduction

Initial studies in several animal models [2, 18, 19, 24] and lately in a phase III trial in kidney transplant recipients, have shown that sirolimus is a potent and effective immunosuppressive drug for the prevention of acute rejection [13]. Unlike cyclosporine and tacrolimus, sirolimus inhibits immune responses by interfering with intracellular signaling by mitogenic cytokines [1]. Sirolimus also effectively prevents intimal thickening in rat heart and arterial allografts and in rat and pig vessels that have undergone balloon catheter injury [6, 8, 9, 17]. Recently it has been shown in rats that sirolimus even halts the progression of pre-existing intimal thickening [23]. This profile for sirolimus is unusual for an immunosuppressant, and its effects may be partially explained by in vitro studies that show sirolimus, but not tacrolimus, inhibits growth factor-stimulated smooth

muscle proliferation and migration [3, 16, 22]. Thus, unlike most other immunosuppressants, sirolimus inhibits the proliferation of both immune and mesenchymal cells, suggesting it may be particularly well suited for the control of graft vascular disease (GVD) in clinical transplantation.

Since rodent models over-predict the efficacy of therapies to inhibit GVD, a non-human primate model for GVD is required to assess more precisely the potential clinical value of novel immunosuppressants. We have now developed a new model in which untreated monkey recipients of aortic allografts (but not autografts) develop lesions that are histologically indistinguishable from human allograft vasculopathy [10]. The progressive increase in graft intimal thickening is sensitively quantified by repeated intravascular ultrasound (IVUS) [12].

The goals of this present study were to compare the efficacies for inhibition of intimal thickening of sirolimus and cyclosporine with each other in monkey aortic allograft recipients relative to untreated recipients and to assess the correlations of the efficacies with trough levels.

Materials and methods

Experimental design

Eighteen cynomolgus monkey recipients of aortic allografts were divided into six untreated controls, six animals treated with sirolimus, and six animals treated with cyclosporine daily from postoperative day 45. The monkeys were killed on day 105, except one cyclosporine-treated monkey that was killed after day 63 after intimal dissection in the graft. Liquid sirolimus solution (10 mg/ml) was mixed with monkey gruel and was administered orally by gavage once daily at a dose of 1.5 mg/kg per day during the first week and 2 mg/kg per day subsequently so that all monkeys received the same dose on a given day. In contrast, liquid solution cyclosporine (doses 2–8 mg/kg per day administered daily IM) were adjusted individually to maintain 24-h trough concentrations at a target range from 300 to 450 ng/ml. Both drugs were generous gifts from Dr. Suren Sehgal, Wyeth-Ayerst Research, Princeton, N.J., USA.

Animals

Healthy male *Macaca fascicularis* (cynomolgus) monkeys free from herpes-B, hepatitis, tuberculosis, simian immunodeficiency virus, simian retrovirus, and simian T-lymphotrophic virus were obtained from Charles River Laboratories (BRF, Inc, Houston, Texas, USA) and quarantined for 6 weeks, during which time the donor and recipient pairs were ABO matched. Mixed lymphocyte reaction typing (stimulation index over 2.5) was used to insure that histoincompatible pairs were chosen. Water and primate chow was provided ad libitum and the animals were kept in temperature and humidity controlled facilities. The animals were cared for according to the standards of the US Public Health Policy of the Humane Care and Use of Laboratory Animals (PHS Manual, Ch. 143) and the guide for the Care and Use of Laboratory Animals, NIH Publication No 8523, revised 1985. The study protocol was approved by the Institutional Laboratory Animal Committee.

Anesthesia, surgical procedure and postoperative care

For surgical procedures, the animals were anesthetized with ketamine hydrochloride 10 mg/kg SC and atropine sulfate 0.01 mg/kg IV. After endotracheal intubation, the animals were ventilated with 40% oxygen and 1.5–2% isoflurane at a tidal volume of 15 ml/kg. Two monkeys were operated on simultaneously by two surgical teams and the segments of abdominal aortas were exchanged between the pairs of monkeys. For postoperative pain, buprenorphine hydrochloride (Buprenex) 0.03 mg/kg was administered IM every 6–8 h.

IVUS studies

Intravascular ultrasound (IVUS) was performed transfemorally on days 7, 21, 42, 63, 84, and 105 after transplantation. A 30 MHz, 2.9 F mechanical catheter ultrasound system (CVIS/BSC) containing a motorized pullback device was used. More than 60 cross-sections were assessed from each graft and the adjacent aorta, and every fourth cross-section (approximately 15 sections/graft per study at 2 mm distance) was digitized morphometrically to quantify vessel areas (VA), luminal areas (LA), intimal areas (IA = VA-LA) and intimal indexes (II). For the present study, the only IVUS measures used were from the six consecutive cross-sections for each graft that were determined to have the least intimal thickening (approximately the middle of the graft) on day 42. In addition to changes in II from one time to another, the absolute increases in II over different time periods were calculated to determine the progression of intimal thickening in grafts in all animals in each of the three groups.

Sirolimus concentrations

EDTA anticoagulated whole blood trough (24-h) sirolimus concentrations were assessed weekly or every other week using a modification of the HPLC/electrospray-MS assay described by Streit et al. [25]. A mean of three consecutive trough levels were determined at the ends of treatment weeks 1, 2 and 8. Altogether 11 samples per monkey were analyzed for sirolimus levels. Weighted means were calculated from the trough levels taking the time range, which each value represented, into consideration. The values at 1, 2, 7 and 8 weeks represented 1 week each, the measurements at 4 weeks represented 2 weeks.

Cyclosporine concentrations

Cyclosporine trough concentrations in whole blood were measured twice a week using an assay based on HPLC/HPLC-electrospray mass spectrometry (LC/LC-MS). Cyclosporin D (a kind gift of Novartis Pharma AG, Basle, Switzerland) was used as an internal standard.

Statistical analysis

SPSS software for Windows package (SPSS Inc. Chicago, Ill., USA) was used for statistical analysis. Levels of significance for differences in IVUS measurements among groups were determined by the one-way ANOVA using Tukey's post hoc test to specify the differences between any two groups. Linear regression was used to determine the correlation between IVUS parameters and the sirolimus or cyclosporine blood levels. P-values < 0.05 were considered significant.

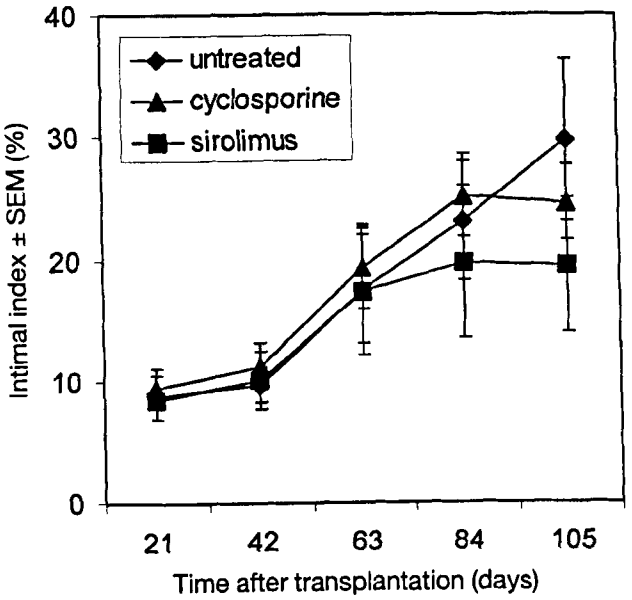

Fig. 1 Mean intimal indexes (± SEM) in the midgraft (six consecutive cross-sections in the middle of the graft with least graft vascular disease on day 42) as assessed by serial intravascular ultrasound at 3 week intervals. When all three groups were compared, the difference in progression of intimal thickening from day 63 to 105 was statistically significant between untreated and sirolimus-treated monkeys ($P = 0.013$, one-way anova, $P = 0.012$ Tukey's post hoc test). ◆ Untreated, ▲ cyclosporine, ■ sirolimus

Results

Intimal indexes before drug treatment

The midgraft II on day 42 (untreated 10 ± 1%; cyclosporine-treated 11 ± 2%; sirolimus-treated 10 ± 2%) did not differ significantly among the groups (P = NS, one-way ANOVA) and the change in II from day 21 to day 42 was also similar among the groups (P = NS, one-way ANOVA). Figure 1 shows that the II had not increased substantially on day 42 compared to the baseline on day 21 and that all groups had similar II before treatment was begun in two groups on day 45.

Effect of drug treatment on inhibition of increases in intimal indexes

The II increased significantly from day 42 to day 105 in grafts in untreated and cyclosporine-treated monkeys ($P = 0.013$, $P = 0.001$, respectively, paired t-test), but not in the sirolimus-treated monkeys (Fig. 1). The changes in II from day 42 to day 105 among all three groups did not reach statistical significance (P = NS, one-way ANOVA), since II increased in all three groups from day 42 to day 63. However, the change in II from day 63 to day 105 was significantly greater in the untreated monkeys when compared to the sirolimus-treated monkeys ($P = 0.013$, one-way ANOVA, $P = 0.012$, Tukey's post hoc test). Other pairwise comparisons did not reach significance, indicating that the change in II for monkeys treated with cyclosporine could not be differentiated from untreated monkeys and that with these small samples we could not discriminate between the efficacies of sirolimus and cyclosporine for this particular measure of intimal thickening. The mean absolute changes in the midgraft II ± SEM from day 63 to 105 were 12 ± 3%, 5 ± 1%, and 2 ± 2% for untreated, cyclosporine-treated and sirolimus-treated monkeys, respectively.

Sirolimus and cyclosporine blood levels

Figure 2 shows the mean sirolimus 24-h trough blood concentrations assessed at 1- or 2-week intervals. During the treatment, sirolimus levels ranged between 17 and 90 ng/ml (median, 43 ng/ml). All monkeys were administered the same dose each day (1.5 mg/kg per day for the first week, 2 mg/kg per day thereafter) throughout the study. Cyclosporine levels (Fig. 3) varied between 245 and 1205 ng/ml (median, 560 ng/ml). Cyclosporine was initially administered at a dose of 8 mg/kg per day to reach a target level between 300 and 550 ng/ml. After a cumulative increase in drug level occurred, the cyclosporine dose was reduced, and continued at 4–6 mg/kg per day.

Correlation of changes in intimal indexes with sirolimus and cyclosporine blood levels

The left panel in Fig. 4 shows the relationship between mean sirolimus trough levels and the changes in mean II from day 42 to day 105. There was an inverse correlation between sirolimus concentrations and the changes in II ($r^2 = 0.73$ for linear regression; $P = 0.03$). The mean sirolimus level (35 ng/ml) in the monkey with greatest change in II was below the 99% confidence interval for the mean sirolimus level in the other five monkeys (mean ± 99% CI = 45 ± 8 ng/ml). Cyclosporine trough levels failed to correlate with changes in II in cyclosporine-treated monkeys (Fig. 4, right panel).

Discussion

We have developed the first model of graft vascular disease in non-human primates [12]. In cynomolgus monkey aortic allograft recipients, intimal thickening progresses in the midgrafts in untreated animals. The histological findings in this model are indistinguishable from human allograft vasculopathy [12]. In a previous study

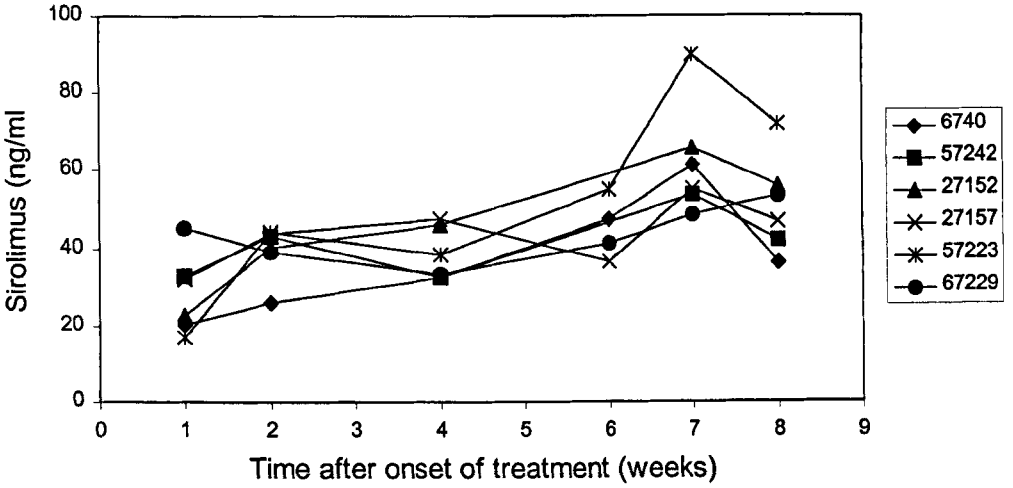

Fig.2 Sirolimus 24-h trough blood concentrations in individual monkeys during the treatment started on postoperative day 45 as monitored by using a modification of the HPLC/electrospray mass spectrometry assay. The values at 1, 2 and 8 weeks are expressed as means of three measurements on consecutive days, whereas all other values are from single measurements. Daily sirolimus dose was the same for all monkeys. Due to interindividual variability, the resulting trough blood concentrations differed 2.5-fold (#6740 low level; #57223, high level). ◆ 6740, ■ 57242, ▲ 27152, × 27157, ✳ 57223, ● 67229

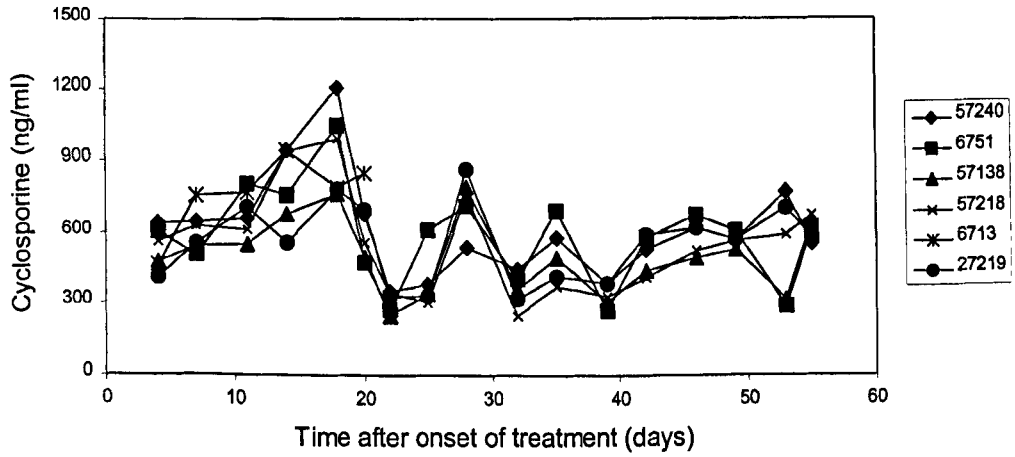

Fig.3 Cyclosporine 24-h trough blood concentrations in individual monkeys as assessed by a specific LC/LC-MS assay twice a week. Cyclosporine doses were individually adjusted to maintain trough blood concentrations in a target range of 300–550 ng/ml. The initial dose of 8 mg/kg per day caused accumulation of the drug and, consequently, the dose for individual monkeys was decreased to 0–4 mg/kg per day after 18 days of treatment. Between treatment days 25 and 60, doses of 4–6 mg/kg per day were required to reach the target level. ◆ 57240, ■ 6751, ▲ 57138, × 57218, ✳ 6713, ● 27219

in rhesus aortic allograft recipients, we have shown that significant intimal thickening develops over a 3-month period only in allografts, but not in autografts [10]. In the present study, delayed sirolimus treatment significantly inhibited increases in intimal index and this measure of the efficacy of sirolimus correlated with trough blood levels.

Treatment was not begun till 45 days after grafting so that acute allograft injury would be unimpeded. Since acute rejection is a well-known risk factor for the development of intimal thickening in patients [11], vasculopathy is believed to be a response to alloimmune injury. We chose to evaluate intimal thickening in the midgraft rather than the whole graft, since intimal thickening in the midgraft was minimal at the time treatment began, thus enabling us to study the effects of these two drugs after acute immune injury had occurred but still in the early phase of GVD.

Our goal was to determine whether trough levels of sirolimus and cyclosporine correlated with inhibition of intimal thickening in aortic allograft recipients. This required that we correlate drug levels with changes in intimal thickening during the period of treatment. Intimal index measures intimal area as a function of graft size (vessel area). Therefore, it compensates for differences in graft size among recipients enabling the changes in intimal thickening from one time to another to be compared and to serve as a means of correlating efficacies with drug levels. For other evaluations, IVUS provides additional means to quantify intimal thickening that are superior to intimal index.

Our data clearly show that despite delayed treatment with sirolimus at a time when acute immune injury would have been expected to occur, this drug was able to inhibit intimal thickening compared to the untreated monkeys. The effect of cyclosporine on inhibition of intimal thickening was less clear. When sirolimus was shown significantly to inhibit the change in intimal index from day 63 to day 105 compared to untreated animals, we did not detect a significant difference between the effects on the changes in intimal index in cyclosporine and untreated animals. This inability to detect a dif-

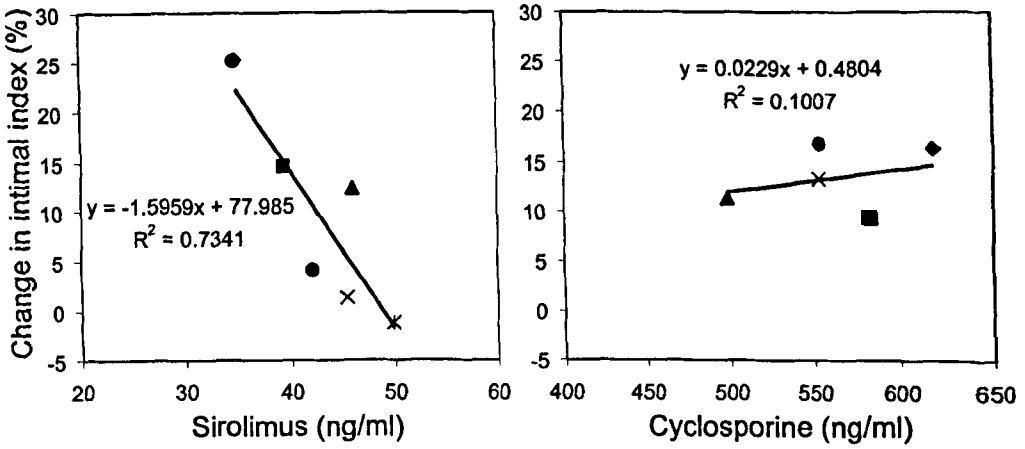

Fig. 4 Absolute changes in the midgraft intimal indexes from day 42 to day 105 versus mean sirolimus 24-h trough blood concentrations during postoperative days 45–100 (*left panel*) and versus mean cyclosporine 24-h trough blood concentrations during postoperative days 45–100 (*right panel*). In the sirolimus-treated monkeys a significant ($P = 0.03$) correlation between the efficacy (less change in intimal index) and trough levels was noticed. In the cyclosporine-treated monkeys the change in intimal index did not correlate with the trough blood concentrations. The symbols of individual animals are the same as in Fig. 3

ference might be explained by the small sample size in each group resulting in low statistical power. This may also explain why we could not detect a significant difference between the changes in intimal index from day 42 to day 105 in the sirolimus- and cyclosporine-treated monkeys.

Nevertheless, sirolimus levels correlated with the changes in intimal index while cyclosporine levels did not. This finding further suggests that sirolimus inhibits the progression of intimal thickening more effectively in aortic allografts after acute immunological injury than does cyclosporine. Sirolimus is not only effective, but its efficacy at lower trough levels than cyclosporine underscores the difference in potencies between these two immunosuppressants.

Others have shown a relationship between sirolimus levels and inhibition of acute rejection and prolongation of graft survival in experimental animals [4, 7, 26]. Since we only measured trough levels of sirolimus, it is important to note that trough levels correlate well with areas under the time-concentration curve (AUC) in patients [14]. Cyclosporine trough levels do not correlate with AUC in any species and this pharmacokinetic characteristic may have limited our ability to note a correlation between cyclosporine trough levels and its efficacy in aortic allograft recipients.

Since one goal of our study was to compare the efficacies of two drugs, the rationale for the choice of doses and levels of each drug are discussed below. Because sirolimus partitions less into formed elements of the blood in rodents compared to non-human primates and man [27], we were concerned that the efficacy of sirolimus for inhibition of intimal thickening after immune (allograft) or mechanical (balloon catheter) arterial injury in rodents was due to the relatively high level of unsequestered drug in rodents. To ensure levels of sirolimus high enough to be effective even after delayed treatment, we aimed at a dose that was highest to be tolerated for a 9-week treatment regimen. The preclinical toxicological data suggested that a daily oral dose of 1.5 mg/kg per day was appropriate and when this dose was well tolerated, we increased the dose to 2 mg/kg. Pharmacokinetic data for sirolimus in monkeys was also used to choose a dose interval of 24 to insure adequate levels throughout the dosing interval.

The trough levels of sirolimus we attained were within the range (10–60 ng/ml) others have used to prolong allograft survival in rabbits [5]. In dog allograft recipients, levels above 10 ng/ml are immunosuppressive [7]. Our sirolimus levels were higher than those that were shown to suppress acute rejection in renal transplant patients [15], but lower levels of sirolimus might have been effective if it had been administered with other immunosuppressants or if we had started treatment with sirolimus on the day of aortic allografting.

To ensure that we were comparing the efficacies of sirolimus and cyclosporine at a corresponding level for inhibition of progression of intimal thickening, we chose doses and levels of cyclosporine that others had shown were immunosuppressive in non-human primates. For example, whole blood trough levels of cyclosporine between 200 and 600 ng/ml prolong graft survival in a highly immunogeneic xenograft model [21]. Others have shown that 8 mg/kg cyclosporine IM prolongs allograft survival in cynomolgus monkeys [20]. For these reasons, we started with a cyclosporine dose of 8 mg/kg IM and adjusted the dose to a target a trough level range of 300–550 ng/ml.

In summary, sirolimus effectively inhibits intimal thickening despite delaying the start of its administration until 45 days after aortic allografting. The efficacy of sirolimus in our model could be due to its known direct inhibition of smooth muscle cell proliferation and migration, its immunosuppressive effects or a combination of all these actions. The superior efficacy of sirolimus compared to the efficacy of high levels of cyclosporine suggests that the combined inhibitory effects of sirolimus on immune cell proliferation and smooth mus-

cle cell proliferation and migration could account for its differentiation from cyclosporine and other drugs that only inhibit lymphocyte proliferation [17]. Additional studies need to be done in our model to examine the efficacy of lower levels of sirolimus, its efficacy when combined with other immunosuppressants and its efficacy when treatment is begun at the time of transplantation.

Our data provide, for the first time, a compelling rationale for the conduct of clinical trials to evaluate the efficacy of sirolimus in organ allograft recipients who are at risk for GVD due to a prior episode of acute rejection that has caused graft injury.

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