T cell receptor stimulated calcineurin activity is inhibited in isolated T cells from transplant patients

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Abbreviations: BMI - body mass index  PBMCs - peripheral blood mononuclear cells
   CsA - cyclosporine A  Tac - Tacrolimus
   IL-2 – interleukin 2  TCR - T Cell Receptor

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Pulmonary, and Renal
ABSTRACT:

The addition of calcineurin inhibitors including cyclosporine A (CsA) and FK-506 (Tacrolimus) to transplant protocols has markedly reduced acute allograft rejection and prolonged patient survival. Although monitoring of serum drug levels has been shown to be a poor indicator of efficacy, there is little data on calcineurin enzymatic activity in humans. Therefore, we measured calcineurin in isolated CD3+4+ T cells from 81 non-transplant controls and 39 renal allograft patients using a 32PO4–labeled calcineurin-specific substrate. A gender difference was observed in the control cohort with activity in males significantly higher than females (1073±134 vs. 758±75 fmol/µg/min). Activity of both groups was comparably inhibited by 5ng/ml Tacrolimus (27±4% vs. 30±4%). Calcineurin is a downstream target of the T cell receptor (TCR). Therefore, activity was measured in isolated T cells following incubation with anti-CD3/CD28 antibodies to stimulate the TCR. Calcineurin activity increased significantly from 1214±111 to 1652±138 fmol/µg/min; addition of either Tacrolimus or CsA (500 ng/ml) blocked CD3/CD28 stimulation. Despite therapeutic levels of Tacrolimus and CsA (mean 11.4 and 172 ng/ml), basal calcineurin activity was significantly higher among renal transplant recipients than controls (1776±175 vs. 914±78 fmol/µg/min). In contrast, anti-CD3/CD28 antibodies failed to stimulate calcineurin activity in transplant subjects. Finally, we found that basal and stimulated calcineurin activities are inversely related. Consistent with this finding, basal activity in resting T cells rose over time post-transplant while stimulation fell (r²=0.785, p<0.05). These data suggest that examination of TCR-stimulated calcineurin activity following renal transplantation may be useful for monitoring immunosuppression of individual patients.
INTRODUCTION:

Calcineurin is a heterotrimeric serine-threonine phosphatase that is composed of a catalytic subunit (A), a regulatory subunit (B) and calmodulin (Rusnak and Mertz, 2000). Calcineurin is unique among phosphatases in that its activity is calcium-dependent and is central to T cell receptor (TCR) signaling and amplification of immune responses. The activation of the TCR complex leads to the release of intracellular calcium and calcineurin-mediated dephosphorylation of transcription factors that regulate IL-2 and other pro-inflammatory cytokines (Macian, 2005).

Cyclosporine A (CsA) and FK-506 (Tacrolimus) are structurally unrelated compounds that form drug-receptor complexes with immunophilins (cyclophilin-18 and FKBP-12 respectively) and potently inhibit calcineurin phosphatase activity. The wide spread use of CsA and Tacrolimus in the past two decades has markedly reduced the frequency of acute allograft rejection and prolonged patients survival. Despite their proven benefits, therapeutic monitoring of CsA and Tacrolimus levels has proven to be a poor clinical indicator of transplant outcomes. Some patients experience rejection in the presence of adequate or even high blood concentrations (Caruso et al., 2001), whereas others develop toxicity even when blood trough concentrations are low (Citterio, 2004; Kahan, 2004). However, in the absence of an alternative means of monitoring calcineurin inhibitor efficacy, current treatment protocols continue to rely upon plasma drug levels for therapeutic monitoring and optimizing immunosuppression.

One potential alternative to plasma drug level monitoring is direct assay of calcineurin activity. However, few studies have directly examined calcineurin activity in T cells or investigated the effects of calcineurin inhibitors on enzyme activity. Previous studies of
calcineurin activity in vivo have focused on issues including pharmacodynamics in response to cyclosporin and tacrolimus (Koefoed-Nielsen and Jorgensen, 2002; Koefoed-Nielsen et al., 2005b; Koefoed-Nielsen et al., 2006; Mortensen et al., 2006) and possible effects of variables including gender and time of day (Koefoed-Nielsen et al., 2005a). In an early study using transplant patients, Batiuk et al used a $^{32}$PO$_4$-labeled calcineurin specific substrate to measure the effects of CsA on calcineurin activity in 30 renal allograft recipients. In vivo measurements demonstrated that calcineurin activity was inhibited by up to 80% one hour after an oral dose of CsA, but only 20-30% within four hours (Batiuk et al., 1997). However, the degree of enzyme inhibition and effect on cytokine production varied greatly between individuals. In a similar study, Pai et al examined the long-term effect of CsA on calcineurin activity in peripheral lymphocytes from bone marrow transplant patients. While CsA initially inhibited calcineurin activity during the first 100 days of transplantation, enzyme activity progressively rose over time and within 6 months was similar to non-transplant controls (Pai et al., 1994).

The aim of this study, therefore, was to compare the effects of CsA and Tacrolimus on calcineurin activity in CD3$^+$/4$^+$ T cells isolated from normal controls and renal transplant patients. In addition, we examined if post-transplant immunosuppression led to changes in calcineurin activity in response to TCR stimulation.
METHODS:

Subject recruitment: Subjects for this study included 81 control volunteers and 39 renal allograft recipients transplanted between 2001 and 2004. For some experiments, a subset of 30 controls was utilized. All participants signed informed consents and completed a brief questionnaire; additional data was obtained by review of patient charts. All data collection, storage, and analyses were carried out with the approval of the Emory University Institution Review Board. Immunosuppressive protocols in use at Emory University from 2001 to 2004 were based on triple drug therapy including glucocorticoids (prednisone), calcineurin inhibitors (Sandimmune or Tacrolimus) and mycophenolate mofetil.

T cell isolation and treatment: Approximately 40mLs of heparinized blood were collected and T cells were isolated using Prepacyte SC reagent (BioE, St. Paul MN). Samples were treated with Vitalyse (BioE) to remove erythrocytes. T cells were pelleted by centrifugation, washed with 1X phosphate-buffered saline, and resuspended in RPMI 1640 containing pen/strep antibiotics, 10% fetal calf serum, 2mM l-glutamine, 25mM glucose, and 1mM sodium pyruvate. Isolated cells were characterized by flow cytometry as 98-99% CD3+CD4+. As indicated, isolated T-cells were separated into aliquots and stimulated for 30 minutes with anti-CD3/CD28 antibodies (10µg/ml each) (BD Biosciences, San Jose, CA).

Calcineurin assay: Calcineurin activity was determined using an in vitro assay based on the method published by Fruman et al (Fruman et al., 1996) and as previously described (Lea et al., 2002). Following treatment, isolated lymphocytes were pelleted, re-suspended in reaction buffer
(100µM Tris, 250 µM KCl, 10mg/ml BSA, 5mg/ml DTT, pH 7.5), and lysed by three cycles of freeze/thawing in liquid nitrogen and a 37°C water bath. 10µg of protein was used for determination of calcineurin activity. Reactions were performed in triplicate, normalized for background, and then a final mean value was determined.

**Measurement of Interleukin-2:** Isolated T cells were treated as described above and the culture medium collected at the end of the incubation period. IL-2 was then measured from the conditioned medium of control or anti-CD3/CD28 antibody-treated T cells using a Panomics Cytokine array according to the manufacturer’s instructions (Panomics, Fremont CA). Data obtained were the fold difference in duplicate measurements of IL-2 for each sample compared to the internal controls.

**Statistics:** All statistical calculations were carried out using GraphPad Prism scientific graphing and analysis software. Paired T tests and repeated measure analysis of variance (ANOVA) were used as indicated to compare multiple treatments of individual samples. For comparison of 3 or more groups, ANOVA (or repeated measure ANOVA, as appropriate) was used in conjunction with Tukey’s post-test. Comparison of two sets of variables was completed by two-way ANOVA as indicated. All results were considered significant if p<0.05.
RESULTS:

To compare calcineurin activity in circulating T cells, blood was obtained from 81 normal controls and 39 patients with functioning renal allografts. Patients undergoing bone marrow transplantation or other forms of solid organ transplantation were excluded. All normal controls were reported as healthy and free of hypertension, diabetes or chronic renal disease. The demographics of renal transplant patients and normal controls including age, height, weight, gender and racial identification are shown in Table 1. Both control and transplanted cohorts were approximately 50% male and 50% female. The average age of controls was 35.3, and the average body mass index was 27.5. The average age and BMI of the post-transplant patients were slightly higher than the control group at 47.5 years and 28.6, respectively. Both groups were racially diverse with about half Caucasians and half African Americans.

The duration of renal transplantation ranged from 1 month to 14 years (mean 22 months). 20 (51%) were transplanted within one year of study enrollment, while the remaining 19 (49%) patients were greater than one year post-transplant. Two patients had received a prior transplant. All transplant patients were currently taking calcineurin inhibitors with 31 taking Tacrolimus and 8 receiving CsA (Sandimmune). Plasma levels of Tacrolimus and CsA were within therapeutic ranges and averaged 11.4 and 172 ng/ml, respectively.

**Calcineurin activity in the control cohort.** To ensure that calcineurin activity was measured in a uniform population of cells, experiments were performed in isolated lymphocytes that were enriched for CD3+/4+ T cells using erythrocyte lysis, gradient centrifugation, and
Prepacyte SC reagent (BioE, St. Paul MN). Flow cytometric analyses confirmed that over 98% of isolated lymphocytes were viable CD3+/4+ T cells (data not shown).

First, T cells were isolated from control subjects and were then treated in vitro with vehicle (DMSO) or 5µg/ml Tacrolimus for 15 minutes. Fig 1 shows that Tacrolimus significantly reduced calcineurin activity from 913±6 to 662±6 fmole/µg protein/min (p<0.001). Although previous studies have reported no effect of gender on calcineurin activity (Koefoed-Nielsen et al., 2005a), females in our control cohort had statistically lower levels of activity compared to males (1073±133M vs 758±75F fmole/µg protein/min (p<0.05). Despite differences in basal activity, both male and female subjects exhibited equal sensitivity to Tacrolimus (27±4% inhibition vs 30±4% respectively).

Next, the effect of stimulating the T cell receptor (TCR) with anti-CD3/CD28 antibodies on calcineurin activity was examined. Isolated T cells were treated with a variety of stimuli including calcium ionophore, anti-CD3 antibody, phorbol myristoleic acid (PMA) and anti-CD3/CD28 antibodies. Anti-CD3/CD28 treatment elicited the maximal response (data not shown) and was chosen for further study. As shown in Fig 2A, calcineurin activity increased significantly from 1214±111 to 1652±138 fmols/µg/min (p<0.001) in the control group. Pretreatment with either Tacrolimus or CsA (5µg/ml) completely blocked the rise in calcineurin activity. As a functional marker for TCR activation, IL-2 production was measured in the conditioned medium from control and anti-CD3/CD28 treated cells. Fig 2B shows that there was a statistically significant increase in IL-2 release. There were no gender differences in the activation of calcineurin or the inhibitory effect of Tacrolimus or CsA.

In addition to gender, the influence of other demographic variables on basal and TCR-stimulated calcineurin activity was examined by multi-variate analysis. There was no significant
correlation between calcineurin activity and age, body mass index (BMI), or race in the control cohort (Table 2.).

**Calcineurin activity in post-transplant subjects.** Calcineurin activity was measured in isolated T cells from 39 post-renal transplant subjects. When compared to normal controls, calcineurin activity was significantly higher in renal transplant patients (1776±175 vs. 914±78 fmol/µg/min) (p<0.001) (Fig 3). In contrast to control subjects, calcineurin activity in male transplant recipients was lower compared to females (p<0.01). As a result, a significant increase was observed when female control and transplant patients were compared while the difference between male control and transplant subjects did not reach significance.

Next, isolated T cells from the transplant cohort were stimulated with anti-CD3/CD28 antibodies. In contrast to TCR stimulation in controls, anti-CD3/CD28 treatment failed to increase calcineurin activity in the transplant cohort (Fig 4A and 4B). Moreover, the mean increase in calcineurin activity for the control group was significantly higher at 36% compared to only 3% for the transplant group (Fig 4C). Consistent with a decrease in TCR activity, IL-2 production in treated T cells from transplant patients was significantly reduced compared to controls (Fig 4D).

Increased basal calcineurin but decreased TCR-stimulated calcineurin activity in transplant subjects suggests that basal and stimulated calcineurin activities may be inversely related. To examine this, basal and stimulated calcineurin were compared by linear regression. Fig 5A and Fig 5B shows that there is a significant inverse association between basal and TCR stimulated calcineurin in both the control and transplant cohorts. In addition, we examined the correlation between basal and stimulated calcineurin activity and changes over time following
transplantation. Baseline calcineurin activity rose over time following transplantation and TCR stimulation decreased (Fig 6). Basal calcineurin activity at one month was 1491±316 fmoles/µg/min rising to 2117±150 fmoles/µg/min at one year and 3834±987 fmoles/µg/min by year three. TCR stimulation decreased over the same period. There was no difference in trough Tacrolimus levels between any of the groups.

Finally, basal and TCR stimulated calcineurin activities were compared to various parameters including age, race, BMI, and Tacrolimus plasma level. There was an inverse correlation between basal calcineurin and BMI. No other parameter reached statistical significance.
DISCUSSION:

Addition of the calcineurin inhibitors CsA and Tacrolimus to immunosuppression protocols has reduced acute rejection rates and prolonged renal allograft survival. However, despite the contribution of these agents to transplant outcomes, it remains to be determined why plasma drug levels fail to predict either rejection or toxicity. Moreover, little is understood about the effects of chronic immunosuppressive therapies on calcineurin activity in lymphocytes from allograft recipients.

To better utilize calcineurin activity as a means of monitoring the efficacy of immunosuppressant drugs, it is essential to identify reproducible changes in activity between control and transplant populations. Reports in the literature to date have not identified an aspect of calcineurin activity that is consistently altered with CsA or Tacrolimus therapy. The current study is therefore the first, to our knowledge, to identify a novel activity that is significantly inhibited in transplant patients. In addition to this important observation, data from this study offer several new insights into calcineurin activity in humans.

First, we found that gender influenced baseline calcineurin activity. Among normal controls, basal calcineurin activity was significantly lower in T cells from females compared to males. The mechanism for reduced calcineurin activity among females is unknown. One possibility may be related to estrogen as previous studies have shown that estrogens can alter protein expression and calcineurin activity in cultured T cells (Rider et al., 2000). Contrary to our study, however, estrogen stimulated expression and activity of calcineurin in vitro. Interestingly, there are no studies that the authors are aware of investigating the effect of testosterone on calcineurin expression in human T cells. It is also possible that additional co-
factors may influence gender differences in calcineurin activity. For example, our control and transplant cohorts are racially diverse with roughly 50% African Americans in each group. Racial influences on gender could also explain why our study identified differences while data from Koeford-Nielsen et al (Koefoed-Nielsen et al., 2005a), completed in a Danish population, did not.

Next, somewhat paradoxically, we report that basal calcineurin activity is increased rather than decreased in transplant patients compared to controls. While there is overlap between the two groups, the finding was highly significant and suggests that the primary change in calcineurin activity with chronic CsA or Tacrolimus treatment is not a sustained decrease in basal levels of the enzyme. Supporting this, Pai et al reported that while calcineurin activity was lower in the first 100 days following bone marrow transplant, after 6 months there was no difference in basal calcineurin activity between CsA treated patients and age matched controls (Pai et al., 1994).

Despite the importance of stimulated calcineurin activity in the dephosphorylation of NFATc and the amplification of an immune response, few studies have examined calcineurin activity in activated lymphocytes and none, to our knowledge, have compared activated calcineurin between control and transplant populations. To address this question, we measured calcineurin activity in CD3+/4+ T cells isolated from normal controls and renal transplant patients stimulated with CD3/CD28 co-stimulatory antibodies. Stimulation of the TCR in transplant patients with therapeutic blood levels of Tacrolimus or CsA did not increase calcineurin activity above basal levels, while enzyme activity in normal controls increased by roughly 40%. These data suggest that TCR-stimulated calcineurin may be a more sensitive indicator of calcineurin inhibitor efficacy than basal levels of enzyme activity.
Our data identified a decrease in TCR-stimulated calcineurin in conjunction with an increase in basal activity in transplant subjects suggesting that there may be a relationship between basal enzyme activity and stimulation. In control and transplant cohorts, a significant, inverse relationship was identified. One explanation for this finding could be that increased basal activity is a compensatory response to loss of TCR stimulation and, in a chronic setting, drugs such as CsA and Tacrolimus act preferentially to inhibit acute activation of calcineurin rather than reduce baseline levels. Future studies examining protein levels of calcineurin could be useful in addressing this possibility. An alternative explanation for the relationship between basal and stimulated calcineurin is that immune challenges such as organ transplant may provoke a chronic elevation in T cell activation and, consequently, of calcineurin activity. Acute challenge of the TCR may then produce no additional increase. In support of this, Fig 5 shows that only 10% of controls had basal calcineurin levels greater than 1700 fmol/µg protein/min. In contrast, almost half of transplant subjects had calcineurin activities >1700. While further studies are needed to understand this possibility, it is clear that monitoring of TCR-stimulated calcineurin may offer a novel alternative to plasma drug levels for therapeutic monitoring of calcineurin inhibitor efficacy.

Supporting the possibility that TCR-stimulation of calcineurin may be physiologically relevant in transplant populations, we found significant correlations between the duration of transplantation and calcineurin activity. Basal calcineurin activity gradually declined and TCR-stimulation increased in subsets of allograft recipients who were 1, 2, and 3 years post-transplant. This would be consistent with a model of gradually declining immune activation and increased graft stability. A similar observation was reported by Mortensen et al who noted that basal
calcineurin activity rose by 40% over a period of 5 years in 20 renal transplant patients (Mortensen et al., 2006).

In conclusion, our data provide a novel paradigm for calcineurin response in T cells of transplant patients. Both basal levels of activity as well as response to TCR stimulation are altered in allograft recipients compared to normal controls. Moreover, these factors are likely related although the mechanism has yet to be defined. Additional prospective studies are needed to determine whether routine measurements of T cell calcineurin activity will enhance monitoring of calcineurin inhibitor efficacy and facilitate the development of individual immunosuppressive protocols.
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REFERENCES:


FOOTNOTES:

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LEGENDS FOR FIGURES:

**Figure 1. Basal and Tacrolimus-inhibited calcineurin activity in normal controls.** T cells were isolated from healthy control subjects (N=81). Samples were then lysed and calcineurin activity measured at baseline and after the in vitro addition of Tacrolimus (Tac) (5.0 ng/ml). Data shown in each column are the mean ± SE calcineurin activity for each group. Tacrolimus significantly decreased calcineurin activity in the whole group as well as in female and male subsets (p<0.001, Two-way ANOVA). Calcineurin activity in T cells isolated from female controls was significantly lower than males (p<0.05, ANOVA, Tukey’s post-test).

**Figure 2. T cell receptor stimulation increases calcineurin activity in normal controls.** A) T cells isolated from a subset of control subjects (N=30) were separated into 4 aliquots which were then incubated with DMSO (control), anti-CD3/CD28 antibodies (10µg/ml each), Tacrolimus (Tac) + CD3/CD28, or CsA+CD3/CD28. Samples were then lysed and calcineurin activity determined. Data shown are the mean ± SE calcineurin activity following each treatment for the whole group as well as female and male sub-populations. Anti-CD3/CD28 treatment resulted in a significant increase in calcineurin activity and pre-treatment with either Tacrolimus or CsA was effective at blocking the induction (*p<0.05, **p<0.01, Two-way ANOVA). There was no gender difference in stimulated calcineurin activity or inhibition by Tacrolimus or CsA. B) IL-2 release into the conditioned medium of control and anti-CD3/CD38 cells was measured using a Panomics cytokine array. Anti-CD3/CD28 treatment resulted in a significant increase in IL-2 in the control group (**p<0.01). There were similar changes in both females and males, although the data did not reach significance.
Figure 3. Increased basal calcineurin activity in transplant subjects. T cells were isolated from 39 post-renal transplant subjects and basal activity was compared to controls. Data shown are the mean ± SE calcineurin activity of controls and transplants as well as female and male subsets of each cohort. Basal calcineurin activity was significantly higher in transplant subjects as a whole and in the female subset (**p<0.01, Two-way ANOVA). Male allograft recipients had lower calcineurin activity compared to females (*p<0.05, ANOVA, Tukey’s post-test).

Figure 4. Calcineurin activity in T cells isolated from transplant subjects is not increased in response to T cell receptor stimulation. Calcineurin activity in control and anti-CD3/CD28 treated T cells isolated from A) control (N= 30) and B) post-transplant (N=39) subjects was compared. T cell receptor stimulation resulted in a significant increase in controls (p<0.001), but not transplants (paired T-test). C) The percent increase in calcineurin activity was determined for control subjects and transplant patients. Data shown are the mean ± SE percent change in stimulate calcineurin activity for the control and transplant groups. The mean percentage of stimulated calcineurin activity was significantly less in transplant subjects compared to controls (** p<0.001, Student’s T-test). D) IL-2 release into conditioned medium of treated cells was compared for control and transplant cohorts. There was a statistically significant decrease in the amount of IL-2 released by T cells isolated from transplant patients (**p<0.01).

Figure 5. Basal and T cell receptor-stimulated calcineurin activity are inversely related. Basal calcineurin activity and anti-CD3/CD28 stimulated activity were compared. There is a statistically significant inverse relationship between basal calcineurin activity and the percent
stimulation in response to T cell receptor stimulation in both the control (A) and transplant (B) groups ($r^2 0.136$, $p<0.01$ and $r^2 0.154$, $p<0.05$ respectively, linear regression).

**Figure 6. Correlation between basal calcineurin activity and time since renal transplantation.** Basal calcineurin activity and response to TCR stimulation were compared in renal allograft recipients and grouped by time since transplantation. Calcineurin activity in patients 1-2 months post-transplant was compared with patients of 3, 12, 24 and 36 months transplant duration. There was a significant positive correlation between basal calcineurin activity and duration of transplantation ($p<0.05$) and a significant inverse correlation between response to T cell receptor stimulation and duration of transplantation ($p<0.05$).
Table 1. Demographic characteristics of study participants. Study participants completed a questionnaire and self-reported gender, race (AA = African American), age, and body-mass index are reported for the control and transplant cohorts.

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Table 2. Calcineurin activity and patient demographics

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Basal and TCR-stimulated calcineurin activity and study participant characteristics were analyzed by Pearson Correlation. Data shown are the Pearson r / p value (if less than 0.05).
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.

Basal calcineurin activity (fmol/μg/min)

Time since transplant (months)

% TCR-stimulated calcineurin activity

Basal

TCR-stimulated

p<0.05