Inhibition of Calcineurin Phosphatase Activity in Adult Bone Marrow Transplant Patients Treated With Cyclosporine A

By Sung-Yun Pai, David A. Fruman, Traci Leong, Donna Neuberg, Thomas G. Rosano, Carol McGarigle, Joseph H. Antin, and Barbara E. Bierer

In vitro studies have demonstrated that cyclosporine A (CsA) and FK506 are potent immunosuppressive agents that have greatly improved the outcome of organ transplantation. First introduced in 1979, CsA was shown to prolong kidney allograft survival, and its use has since been extended to a variety of solid organ transplant settings. In bone marrow transplantation (BMT), CsA has also been shown to be effective in preventing the development of graft-versus-host disease (GVHD), a multisystem disorder mediated by donor T cells. Both acute and chronic GVHD are major causes of morbidity and mortality that limit the success of BMT. More recently, FK506, a structurally unrelated macrolide compound with a spectrum of action similar if not identical to that of CsA, has also been used for the treatment of GVHD.

CsA and FK506 interfere with a number of calcium-dependent T-cell activation events. CsA binds to a family of intracellular receptors termed cyclophilins (CyP), while FK506 binds to a family of receptors termed FK-binding proteins (FKBP). The complexes of CsA bound to CyP and FK506 bound to FKBP act in turn on a common intracellular target, calcineurin, a serine-threonine phosphatase the activity of which requires the binding of calcium. Both biochemical and molecular studies have confirmed that inhibition of calcineurin activity by CsA and FK506 measured in vitro correlates closely with the inhibition of lymphokine gene activation (eg, interleukin-2) and other downstream events in T-cell activation. Thus, CsA and FK506 form drug-binding protein complexes that inhibit the phosphatase activity of calcineurin, interfering with the dephosphorylation of critical signaling molecules and abrogating T-cell function.

The role of inhibition of calcineurin in clinical immunosuppression has yet to be defined. Our first goal was to determine whether administration of CsA in vivo is associated with inhibition of calcineurin activity, as has been shown in vitro. If this were the case, direct measurement of calcineurin activity may prove useful as an alternative to measurement of trough CsA levels for assessing the adequacy of immunosuppression in BMT, as well as in solid organ transplantation. The study of calcineurin activity in vivo may also have relevance for the clinical management of GVHD. Current therapy of GVHD is aimed at suppressing donor T-cell function. Inadequate suppression of calcineurin activity despite prophylactic CsA treatment would result in T-cell alloreactivity, promoting the development of GVHD. For example, chronic CsA administration might result in downmodulation of intracellular CyP expression, leading to a deficiency of inhibitory complexes and the inability to inhibit calcineurin activity. If this were the case, changing the immunosuppressive regimen from CsA to FK506, which acts via a distinct receptor (FKBP) to inhibit calcineurin activity, could be therapeutically beneficial. Alternatively, if calcineurin activity is adequately suppressed in patients with GVHD, simply increasing the dose of CsA or changing to FK506 would not be expected to ameliorate the disease.

To study the importance of calcineurin activity in clinical immunosuppression, the calcineurin activities of blood mononuclear cells isolated from 62 adult BMT recipients were determined. Patients were stratified according to time since transplant, presence or absence of CsA in plasma, and presence or absence of GVHD. Of 62 patients, 33 were taking CsA and 29 were not. Early posttransplant (<100 days), the calcineurin activity of patients on CsA was significantly lower than that of patients not on CsA (P = .0004) and than that of normal volunteers (P < .0001). Similarly, late posttransplant (>100 days), the calcineurin activity of patients taking CsA was inhibited compared with normal volunteers (P < .05). The calcineurin activity of patients with acute GVHD who were taking CsA was lower than that of patients on CsA without acute GVHD matched for time post transplant (P = .02). Calcineurin activity in patients on CsA with chronic GVHD was similar to those without chronic GVHD on drug. In conclusion, calcineurin activity is significantly suppressed by in vivo administration of CsA. The lower calcineurin activity of patients on CsA with acute GVHD suggests that CsA-resistant GVHD is not the result of inadequate suppression of calcineurin activity. These data suggest that if inhibition of calcineurin is the only physiologic target of CsA administration, simply increasing doses of CsA or treatment with other inhibitors of calcineurin, such as FK506, would not be expected to ameliorate GVHD.

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posttransplant, and calcineurin activities were compared based on the presence or absence of CsA and the presence or absence of GVHD.

MATERIALS AND METHODS

Cells. Peripheral blood samples were collected from 62 adult BMT recipients from the Brigham and Women's Hospital, Boston, MA, and from 12 normal healthy volunteers, after informed consent had been obtained, in accord with the Human Research Committee of the Brigham and Women's Hospital. When possible, multiple samples from the same patient were collected at varying times posttransplant. Clinical data were recorded, including the presence or absence of acute (grade 1 or greater) or chronic GVHD,\textsuperscript{14,15} presence or absence of CsA, dose prescribed, days posttransplant, and other medications. For each patient, 1 mL of plasma was frozen for the later determination of CsA level by radioimmunooassay (RIA) (see below). Peripheral blood mononuclear cells (PBMC) were isolated from the remainder of the sample by Ficoll density gradient centrifugation (specific gravity, 1.077; Organon Teknika, Durham, NC) and cells not used for the calcineurin assay were frozen for analysis of calcineurin B and cyclophilin A (CyPA) protein expression by immunoblotting techniques (see below).

The human T-leukemia cell line Jurkat (American Type Culture Collection, Rockville, MD) was cultured in RPMI 1640 medium (GIBCO, Grand Rapids, NY) supplemented with 10% heat-inactivated fetal calf serum (Sigma Chemical, St Louis, MO), 2 nmol/L L-glutamine (Whitaker, Walkersville, MD), 100 μg/mL penicillin (GIBCO), 100 μg/mL streptomycin (GIBCO), 10 mmol/L Hepes (M.A. Bioproducts, Bethesda, MD), pH 7.2, and 50 μmol/L 2-ME (Sigma) (termed RPMI-10% FCS).

Calcineurin assay. Calcineurin assays were performed essentially as described previously.\textsuperscript{10,12,20} Briefly, hypotonic lysates\textsuperscript{10} were prepared using PBMC isolated from BMT patients. After two freeze-thaw cycles, the lysates were stored in liquid nitrogen; storage for up to 2 weeks did not result in any demonstrable loss of calcineurin activity (data not shown). Biweekly, lysates were thawed, centrifuged to remove nuclei, and assayed for protein content by the Bradford method.\textsuperscript{21} Lysates with high protein concentration were diluted with hypotonic lysis buffer to ensure a relative excess of exogenous substrate. Assays were performed in duplicate using 20 μL of lysate, 20 μL of assay buffer, and 20 μL of 15-μmol/L \textsuperscript{32}P labeled substrate. The assay buffer contained 1.5 μmol/L 2-acetaic acid (500 nmol/L final) to inhibit the activity of phosphatase 2A.\textsuperscript{22} In addition, magnesium was excluded from the assay buffer to minimize the activity of phosphatase 2C.\textsuperscript{22} Reactions were terminated after 15 minutes at 30°C, and free phosphate was isolated using Dowex cation-exchange columns (BioRad, Richmond, CA). Calcineurin activity was measured as picomoles phosphate released per minute, multiplied by the dilution factor when appropriate. On each day that patient samples were collected and on the day of each assay, normal PBMC and Jurkat cells were incubated with exogenous CsA or vehicle control for 1 hour, then assayed for calcineurin activity as above. The calcineurin activities of Jurkat cells fell consistently within the range expected based on previous experience. The activities of CsA-treated mononuclear cells from normal volunteers were equivalent to those of CsA-treated Jurkat cells.

Immunoblotting analysis. Frozen patient-cell samples containing approximately 2 to 5 × 10^7 cells each were lysed with 20 μL of buffer containing 50 mmol/L Tris, pH 8.0, 0.5% Triton X-100, 150 mmol/L NaCl, 50 μg/mL phenylmethylsulfonylfluoride, 50 μg/mL soybean trypsin inhibitor, 10 μg/mL leupeptin, and 10 μg/mL aprotenin. Lysates were incubated for 10 minutes on ice and then centrifuged to remove nuclei. Equivalent amounts of protein from all patients were resolved by 15% sodium dodecyl sulfate–polyacryl-
mide gel electrophoresis. Proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA) and blocked with 4% bovine serum albumin (United States Biochemical, Cleveland, OH). Western blots were performed using mouse monoclonal antibody directed against calcineurin B chain (Upstate Biotechnology, Lake Placid, NY) and rabbit polyclonal serum directed against CyPA (kind gift of Dr Christopher Walsh, Dana-Farber Cancer Institute, Boston, MA). Blots were probed with the appropriate horseradish peroxidase-conjugated secondary antibody, and protein was detected by enhanced chemiluminescence techniques (Amersham Life Science, Arlington Heights, IL).

RIA for CsA. CsA levels were determined from plasma samples using the TDx cyclosporine monoclonal plasma assay (Abbott Laboratories, Chicago, IL) according to the manufacturer's specification without modification.

Statistical analysis.Calcineurin activities obtained from the same patient within a given time period were averaged if the GVHD status of the patient did not change within that time period. The Wilcoxon rank-sum test was used for all comparisons. Testing was not adjusted for multiple comparisons, and only nominal \textit{P} values are presented here.

RESULTS

Patient characteristics. The study group was composed of 62 patients: 33 allogeneic BMT recipients on CsA and 29 patients who were off drug (Table 1). The patients off CsA included 19 who underwent autologous transplants and never received CsA, nine allogeneic transplant patients in whom drug had been discontinued, and one syngeneic patient

<table>
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<th>Characteristic</th>
<th>On CsA</th>
<th>Off CsA</th>
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<tbody>
<tr>
<td>No. of patients</td>
<td>33</td>
<td>29</td>
</tr>
<tr>
<td>No. of observations</td>
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<td>40</td>
</tr>
<tr>
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<td>14/15</td>
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<tr>
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<td>22-52</td>
<td>36</td>
</tr>
<tr>
<td>GVHD status</td>
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<td>Chronic</td>
</tr>
<tr>
<td></td>
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<td>12</td>
</tr>
<tr>
<td></td>
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<td>None</td>
</tr>
<tr>
<td></td>
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<td>14</td>
</tr>
<tr>
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<td>Autologous</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>9</td>
</tr>
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<td>MDS</td>
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<tr>
<td></td>
<td>18</td>
<td>3</td>
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<td></td>
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One patient had acute GVHD (defined as occurring <100 days posttransplant) that later evolved into chronic GVHD (defined as occurring >10 days posttransplant).

Abbreviations: N/A, not applicable; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; ALL, acute lymphocytic leukemia; NHL, non-Hodgkin's lymphoma.
who was never on CsA. Because multiple samples at different time points were collected when possible, there were 60 observations of patients on CsA and 40 observations of those not on CsA. Many patients had only one determination, while in other instances two or three determinations were performed at different time intervals. The male to female ratios and age ranges of the two groups were similar (Table 1). The diagnoses of the two groups differed and reflect the clinical indications for allogeneic and autologous BMT (Table 1). Of 33 patients on CsA, seven had acute GVHD, 11 had chronic GVHD, one had acute GVHD that evolved into chronic GVHD, and 14 did not have either chronic or acute GVHD (Table 1).

Analysis of patients early posttransplant. The calcineurin activity of patients early posttransplant (<100 days) on CsA (mean, 0.9 pmol/min) was significantly less than that of patients off drug (mean, 2.9 pmol/min; P = .0004) and of normal volunteers (mean, 3.3 pmol/min; P < .0001) (Table 2). Because allogeneic patients at risk for GVHD routinely receive prophylaxis with CsA, all of the patients on CsA were allogeneic recipients, while those off drug were autologous recipients. Thus, we cannot eliminate the formal possibility that the type of transplant or the diagnosis for which transplantation was indicated may have had an effect on calcineurin activity, although we consider this unlikely (see below).

Patients on CsA were further categorized according to GVHD status (Table 3). One possible mechanism to explain CsA-resistant GVHD would be persistent calcineurin activity. If, for instance, CyP protein were downmodulated as a consequence of chronic CsA administration, a lack of inhibitory complexes would then lead to inadequate suppression of calcineurin activity. Surprisingly, the calcineurin activity of patients on CsA with acute GVHD (mean, 0.5 pmol/min) was significantly lower, not higher, than that of patients also on drug but with no GVHD (mean, 1.0 pmol/min; P = .02). Decreased measured calcineurin activity could result from either decreased calcineurin polypeptide expression, decreased activity of the enzyme itself, or increased CyP expression in the cells. Therefore, the amount of calcineurin and CyPA protein was determined by immunoblotting analysis. Western blot analyses of lysates failed to show modulation of either calcineurin or of CyPA protein expression in patients with or without GVHD (data not shown). Differences in plasma CsA level may in part account for the greater inhibition of calcineurin activity in patients with acute GVHD (see below).

Analysis of patients late posttransplant. The calcineurin activities of patients taking CsA who were 100 days to 6 months (mean, 1.6 pmol/min) or greater than 6 months posttransplant (mean, 2.3 pmol/min) were significantly lower than that of normal volunteers (mean, 3.1 pmol/min; P = .02 and P < .05 respectively) (Table 2). However, the calcineurin activities of patients greater than 100 days posttransplant on and off CsA were not significantly different (Table 2). If one excludes from analysis patients being tapered off

<table>
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<th>Table 2. Comparison of Calcineurin Activities in Patients on CsA With Patients Off CsA and With Normal Volunteers</th>
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<tbody>
<tr>
<td>Patients</td>
</tr>
<tr>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>&lt;100 Days</td>
</tr>
<tr>
<td>On CsA</td>
</tr>
<tr>
<td>Off CsA</td>
</tr>
<tr>
<td>Normals</td>
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* The calcineurin activity of patients on CsA was compared with that of patients off CsA and that of normal volunteers using the Wilcoxon rank-sum test. Mean values of calcineurin activity in pmol phosphate released per minute are shown, with the number of patients in parentheses. Patients were stratified into those at risk for acute GVHD (<100 days posttransplant) and those at risk for chronic GVHD (100 days to 6 months and >6 months posttransplant).

* Significance ≤ .05 level.

<table>
<thead>
<tr>
<th>Table 3. Correlation of GVHD Status With Calcineurin Activity</th>
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<tr>
<td>Patients</td>
</tr>
<tr>
<td>---------------------------------------------</td>
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<tr>
<td>&lt;100 Days</td>
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<tr>
<td>With GVHD</td>
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<tr>
<td>No GVHD</td>
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* Patients on CsA were stratified into those with acute GVHD, those with chronic GVHD and those with no GVHD. Calcineurin activities in pmol/min of patients with GVHD were compared with those of patients matched for time posttransplant without GVHD. The Wilcoxon test was used for all comparisons and mean values are shown, with the number of patients in parentheses. Note that this analysis includes patients who were on tapering or very low doses of CsA.

* Significance ≤ .05 level.
CsA, the comparison between patients greater than 100 days on and off CsA showed a trend that was similar to the analysis of patients early posttransplant, but this trend did not reach statistical significance (mean, 1.5 pmol/min vs. 3.3 pmol/min; P = .07). Furthermore, the calcineurin activities of patients with and without chronic GVHD were not significantly different (Table 3). However, the number of patients with chronic GVHD was small, and, notably, many of the patients with chronic GVHD were taking very low doses of CsA or were on alternate-day CsA therapy.

**Return of calcineurin activity with increasing time posttransplant.** At less than 100 days posttransplant, calcineurin activity in patients off CsA (mean, 2.9 pmol/min) was not significantly different than that of normal volunteers. When these patients were further subdivided by time posttransplant, we noted that the calcineurin activity of patients 0 to 45 days posttransplant (mean, 1.9 pmol/min) was generally lower than that of normal volunteers (mean, 3.3 pmol/min), while that of patients 45 to 100 days posttransplant (mean, 4.3 pmol/min) was similar to that of normal volunteers (mean, 3.3 pmol/min). The calcineurin activities of patients off CsA who were greater than 100 days posttransplant (mean, 3.5 pmol/min for 100 days to 6 months; mean, 3.0 pmol/min for >6 months) were not different from that of normal volunteers (mean, 3.3 pmol/min).

While patients off CsA early posttransplant (<100 days) were all autologous recipients, patients off CsA late posttransplant (>100 days) also included allogeneic recipients who had been successfully tapered off drug. The calcineurin activity of these nine allogeneic recipients off CsA (mean, 3.3 pmol/min) was not significantly different from that of the 12 autologous patients late posttransplant (mean, 3.3 pmol/min). Thus, we consider it unlikely that the type of transplant correlates with a difference in calcineurin activity, although this confounds our analysis of patients early posttransplant (see above).

**Correlation between CsA level and calcineurin activity.** Figure 1 is a scatterplot of the calcineurin activities of the patients on CsA plotted against plasma CsA level measured by RIA. Most patients with CsA plasma levels greater than 200 ng/mL had calcineurin activities that were less than 1.0 pmol/min. The suppression of calcineurin activity in patients with levels between 200 and 400 ng/mL was similar to that of patients with levels greater than 400 ng/mL. In four determinations, representing three patients, calcineurin activities were found to be greater than 1.0 pmol/min despite CsA levels greater than 200 ng/mL. None of these three patients had either acute or chronic GVHD, which argues against the possibility that a rare patient with GVHD had inappropriately high calcineurin activity. Patients with acute GVHD had significantly higher plasma CsA levels than those without acute GVHD (377 ng/mL vs. 175 ng/mL; *P* = .001). This finding may account for the lower calcineurin activity in patients with acute GVHD (Table 3). It should also be noted that the calcineurin activity of some patients with CsA levels less than 200 ng/mL was as low as that of patients with higher drug levels.

**DISCUSSION**

CsA is an important therapeutic agent for prophylaxis against and treatment of GVHD. We determined the effect of CsA on calcineurin activity in vivo, since the inhibition of calcineurin phosphatase activity appears to be the central mechanism of action of CsA and FK506. Calcineurin activity in the mononuclear cells of patients taking CsA was significantly lower than that of the cells of normal volunteers, both early and late posttransplant (Table 2). Calcineurin activity was also significantly inhibited in patients early posttransplant who were taking CsA compared with transplanted patients not on drug (Table 2). The decrease in calcineurin activity in patients on CsA was not due to a lack of calcineurin protein or modulation of the principal cytoplasmic CsA-binding protein, CyPA (data not shown). The calcineurin activity in patients late posttransplant on CsA was generally lower than that of transplanted patients off CsA (Table 2); a larger sample may be required to reach significance. Taken together, we conclude that CsA administration in vivo correlates with inhibition of mononuclear cell calcineurin activity.

While the preparative regimen for BMT involves the administration of agents that will transiently eliminate immune cell function, patients also exhibit signs of T-cell dysfunction for months after the recovery of CD3+ T cells. Phenotypic abnormalities,24-26 defective interleukin-2 production in the absence of CsA,27 decreased numbers of functional T-cell precursors,28 and decreased proliferative responses29,30 have all been noted following allogeneic BMT. Since calcineurin is an essential signaling intermediate in antigen-dependent T-cell function, deficient calcineurin activity following BMT could contribute to T-cell dysfunction. We found that the calcineurin activity of patients not taking CsA at every time posttransplant was in fact comparable to that of normal volunteers, even at less than 45 days posttransplant (see Results). Because different cell types repopulate the marrow at different rates, the slightly lower activity we observed in patients immediately postengraftment may reflect skewing of the mononuclear cell population towards cell types with...
intrinsically lower calcineurin activity. Thus, the prolonged immune dysfunction seen in patients months after BMT is unlikely to be due to a lack of calcineurin activity.

Our examination of the relationship between calcineurin activity and the presence or absence of GVHD implies that persistent calcineurin activity does not explain CsA-resistant GVHD. Because mature donor T cells mediate GVHD, inadequate suppression of calcineurin activity and therefore preserved T-cell alloreactivity could promote the development of CsA-resistant GVHD. However, the calcineurin activity of patients with acute GVHD was significantly lower, not higher, than that of patients also on CsA but without GVHD (Table 3). Calcineurin and CyP protein was present in lysates of mononuclear cells of all patients; the lower calcineurin activity may instead be secondary to higher levels of plasma CsA (see Results), presumably the result of higher doses of drug prescribed in an attempt to treat GVHD. These findings have important implications for the clinical management of CsA-resistant acute GVHD. If inhibition of calcineurin activity is the only therapeutically relevant effect of CsA, simply raising the dosage of CsA or administration of FK506, an agent that also acts by inhibition of calcineurin activity, is unlikely to ameliorate GVHD.

Raising the dose of CsA to achieve high therapeutic levels has been one therapeutic option for the treatment of GVHD in BMT, and such measures have had anecdotal success. Because of the intrinsic toxicity of CsA, the measurement of drug levels has been used to guide CsA therapy in both BMT and solid-organ transplantation. The recommended therapeutic range for CsA is variable, depending in part on the type of assay performed, as well as on the type of transplant involved. In our study, the calcineurin activities of most patients with CsA levels of 200 ng/mL or greater fell below 1.0 pmol/min (Fig 1). Thus, CsA plasma levels of 200 ng/mL or greater appeared to suppress measurable calcineurin activity in most patients. Since the mononuclear cells of a number of patients with low CsA levels (<200 ng/mL) exhibited low (<1.0 pmol/min) calcineurin activities, it is possible that, in some patients, subtherapeutic doses of CsA may be sufficient to abrogate T-cell function. There was also no apparent increase in calcineurin inhibition at high CsA levels (400 to 600 ng/mL). These findings suggest that raising levels above that needed for maximal calcineurin suppression may contribute to increasing toxicity.

The therapeutic benefit, if any, of increasing the dose of CsA is unlikely therefore to be secondary to an effect on calcineurin activity. At high concentrations, CsA has been shown to have a number of other cellular effects in vitro. For instance, CsA is capable of inhibiting CyP rotamase activity, the cis-trans isomerization around peptidyl-prolyl bonds. The in vivo function of rotamase activity is still unknown, although it is believed to play a role in protein folding. A number of studies have suggested that inhibition of rotamase activity alone does not correlate with immunosuppression. Furthermore, although rotamase activity is affected at concentrations of CsA similar to that needed to inhibit T-cell function, because of the abundance of CyP, micromolar doses of CsA would be required for complete inhibition of all rotamase activity. At such high concentrations, CsA has also been shown to inhibit the proinflammatory effect of CyPs on monocytes and eosinophils. Finally, CsA has been shown to reverse multidrug resistance in tumor cell lines at high concentrations, and may have effects on other transport proteins. However, at these concentrations, toxicity would limit administration and, therefore, the in vivo significance of these other mechanisms of action remains unclear.

In summary, this study begins to illuminate the role of calcineurin in clinical immunosuppression. The findings in this analysis of BMT patients confirm studies performed in vitro on the effect of CsA on calcineurin activity. Investigation of the relationship of calcineurin activity to GVHD suggests that calcineurin is not directly involved in the mechanism of CsA-resistant acute GVHD. In addition to calcium-dependent signals through the T-cell receptor, which activate calcineurin, a second set of costimulatory signals, such as those recruited by engagement of the CD28 receptor, are also required for T-cell function. The CD28 costimulatory pathway is calcium-independent and resistant to inhibition by CsA or FK506; this pathway may be operative in the pathogenesis of GVHD. Our findings thus underscore the need for drugs targeting non–calcineurin-dependent signaling pathways to treat CsA-resistant GVHD.

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