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Cyclophilin A-Deficient Mice Are Resistant to Immunosuppression by Cyclosporine

John Colgan,2* Mohammed Asmal,* Bin Yu,* and Jeremy Luban3*†

Cyclosporine is an immunosuppressive drug that is widely used to prevent organ transplant rejection. Known intracellular ligands for cyclosporine include the cyclophilins, a large family of phylogenetically conserved proteins that potentially regulate protein folding in cells. Immunosuppression by cyclosporine is thought to result from the formation of a drug-cyclophilin complex that binds to and inhibits calcineurin, a serine/threonine phosphatase that is activated by TCR engagement. Amino acids within the cyclophilins that are critical for binding to cyclosporine have been identified. Most of these residues are highly conserved within the 15 mammalian cyclophilins, suggesting that many are potential targets for the drug. We examined the effects of cyclosporine on immune cells and mice lacking Ppia, the gene encoding the prototypical cyclophilin protein cyclophilin A. TCR-induced proliferation and signal transduction by Ppia−/− CD4+ T cells were resistant to cyclosporine, an effect that was attributable to diminished calcineurin inhibition. Immunosuppressive doses of cyclosporine failed to block the responses of Ppia−/− mice to allogeneic challenge. Rag2−/− mice reconstituted with Ppia−/− splenocytes were also cyclosporine resistant, indicating that this property is intrinsic to Ppia−/− immune cells. Thus, among multiple potential ligands, CypA is the primary mediator of immunosuppression by cyclosporine. The Journal of Immunology, 2005, 174: 6030–6038.
would mediate the immunosuppressive effects of cyclosporine in T cells. In contrast, CypA and CypB can mediate calcineurin inhibition when overexpressed in transformed T cells (30), suggesting that both of these proteins have a role in immunosuppression by cyclosporine in vivo.

Mice lacking Pitua, the gene encoding CypA, have augmented Th2-type immune responses attributable to increases in the activity of the Itk, a tyrosine kinase that is inhibited by CypA via recognition of a proline in its Src homology 2 domain (10). In this study, we present analysis of in vitro T cell function and in vivo immune responses that shows Pitua−/− mice are resistant to immunosuppression by cyclosporine.

Materials and Methods

Mice

The 129S6/SvEv Pitua−/− mice have been described (10) and deposited with The Jackson Laboratory Induced Mutant Resource as Stock 5320 (www.jax.org). The 129S6/SvEv Rag2−/− mice were from Taconic Farms. Offspring of Pitua+/− mice obtained by backcrossing seven generations into BALB/c (Taconic Farms) were used as sources of bone marrow for generating allogeneic dendritic cells. Sex-matched, specific pathogen-free mice were from The Jackson Laboratory Induced Mutant Resource as Stock 5320 (JES6-5H4), streptavidin-allophycocyanin, and alkaline phosphatase were from BD Biosciences. Affinity-purified anti-67.1 Ab specific for (JES6-5H4), streptavidin-allophycocyanin, and alkaline phosphatase were from BD Biosciences. Purified Ab to CD3 (145-2C11), CD28 (37.51) and IL-2 (JES6-1A12), FITC-anti-CD4, H2Kd and goat anti-rabbit Ab, PE anti-CD69, H2Dd and CD62L, allophycocyanin anti-CD8, biotinylated anti-CD25 and IL-2 (JES6-5H4), streptavidin-allophycyanin, and alkaline phosphatase were from PerkinElmer Life Science Products. Bone marrow-derived dendritic cells (BMDDCs) were generated as described (32).

Proliferation assays

Cells (2 × 10^6/well) were cultured in 96-well round-bottom plates. To stimulate with plate-bound Ab, wells were incubated for 3 h at 37°C with Ab in PBS and washed with PBS before cells were added. CD4+ T cell proliferation in response to syngeneic or allogeneic cells was assessed by mixing irradiated (2500 rad) BMDDCs (1 × 10^5/well) with purified CD4+ T cells (0.1–3.0 × 10^5/well) in 96-well round-bottom plates. Cyclosporine, methyl-Ile-cyclosporine, and FK506 in oil emulsion were diluted to 1 mg/ml in DMSO, further diluted in RPMI 1640, and added to wells before cells were plated. Cultures were pulsed with [3H]thymidine (1 μCi/well; PerkinElmer Life Science Products) 48 or 72 h (when stimulated with dendritic cells) after plating, and [3H]thymidine incorporation was measured 12 h later using a Top Count (Packard Instrument).

Analysis of IL-2 production by ELISA

Spleen cells (2 × 10^6/well) were plated in 96-well round-bottom plates coated with 10 μg/ml anti-CD3 and containing the indicated amounts of cyclosporine. After 48 h, supernatant from triplicate wells was pooled and assayed for IL-2 by ELISA, according to a protocol from BD Biosciences.

RT-PCR

CD4+ T cells (1 × 10^6/well) were added to wells in a 24-well plate coated with 10 μg/ml anti-CD3 and containing the indicated amounts of cyclosporine. RNA isolation, cDNA synthesis, and real-time RT-PCR analysis were performed, as described (10). Hypoxanthine phosphoribosyltransferase primers were 5’-GGACCTCTCAGGTTGTGGGCTAGAC-3' (forward) and 5’-GACTTACATCTGCGAGGAGCTTG-3' (reverse). IL-2 primers were 5’-CCCTGAGGAGAGGATCAGAAGATCA-3' (forward), 5’-CTCGAGAACATGCGCCGAG-3' (reverse), and 5’-CGCCCAACGACGGCCACAGAATGGAAATATTCCGCG-3' (beacon).

Flow cytometry

Staining and wash buffer was PBS containing 3% FBS and 0.1% sodium azide. Cells were mixed with Ab at concentrations recommended by the manufacturer, incubated for 20 min on ice, and then washed. Analysis was performed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

Surface expression of CD25 and CD69

CD4+ T cells (1 × 10^6/well) were added to wells in 24-well plates coated with 10 μg/ml anti-CD3 and containing the indicated amounts of cyclosporine or FK506. After 20 h, cells were stained with FITC-anti-CD4, PE anti-CD69, biotinylated anti-CD25, and streptavidin-allophycyanin for flow cytometric analysis.
p38 MAPK activation

CD4+ T cells (1 × 10⁶/well) in 24-well plates were incubated for 20 min at 37°C in medium alone or medium containing cyclosporine. PMA and ionomycin were added to 10 and 400 ng/ml, respectively, followed by incubation for 20 min at 37°C. Cells were washed with PBS, fixed using Cytofix buffer (BD PharMingen), and permeabilized using Perm/Wash buffer (BD PharMingen). Anti-phospho p38 MAPK Ab was added, followed by incubation for 20 min on ice. Cells were washed with Perm/Wash buffer, FITC-conjugated anti-rabbit Ab was added to cells in Perm/Wash buffer, followed by incubation for 20 min on ice. Cells were washed with Perm/Wash buffer and analyzed by flow cytometry.

NF-AT dephosphorylation

A total of 2 × 10⁶ lymph node cells was incubated for 20 min at 37°C in medium containing the indicated amounts of cyclosporine. Ionomycin was added to 400 ng/ml, and samples were incubated for 5 min at 37°C. Cells were spun down and resuspended in lysis buffer (5% SDS, 30 mM sodium pyrophosphate, 5 mM EDTA, 2 mM PMSF, 250 μM leupeptin, 100 μg/ml aprotinin, and 2 mM sodium orthovanadate), boiled for 5 min, and then passed through a 26-gauge needle several times. After boiling again for 5 min, 10 μg of protein was resolved by SDS-PAGE, transferred to nitrocellulose, and probed with affinity-purified anti-67.1 Ab. Reactive species were visualized using HRP-conjugated anti-rabbit Ab and a chemiluminescence kit (PerkinElmer).

Cyclosporine treatment

Clinical-grade cyclosporine was diluted fresh daily in PBS and injected i.p. Control animals were injected with PBS alone. Treatment was given daily starting the day before injection of P815 cells.

Assessment of tumor cell clearance and CD62L down-regulation

Mice were injected i.p. with 1 × 10⁶ P815 cells and sacrificed 10 days later. To assess tumor cell clearance, peritoneal exudates were recovered by lavage, counted, and stained with FITC anti-H2Kd and PE anti-H2Dd for analysis by flow cytometry. The number of tumor cells recovered was calculated by determining the percentage of cells staining positive for H2Kd. To assess surface CD62L expression on T cells, splenocytes were stained with FITC anti-CD4, PE anti-CD62L, and allophycocyanin anti-CD8, and analyzed by flow cytometry.

Assay for cytotoxic T cell activity

Mice were injected i.p. with 1 × 10⁷ P815 cells and sacrificed 10 days later. CD8+ cells were isolated from spleen cells using anti-CD8 microbeads. Target cells were labeled by mixing 2 × 10⁷ cells in 0.2 ml of medium with 0.2 ml (200 μCi) of ⁵¹Cr (PerkinElmer Life Science Products), followed by incubation at 37°C for 1 h. Unbound ⁵¹Cr was removed by washing three times with medium. CD8+ cells were mixed with 1 × 10⁴ ⁵¹Cr-labeled P815 or control EL-4 cells (final culture vol 0.2 ml) in flat-bottom 96-well plates. Cells were spun down by brief centrifugation, and plates were incubated for 3 h at 37°C. Supernatant (20 μl) was removed and analyzed for ⁵¹Cr using a Top Count (Packard Instrument). Percent specific lysis was calculated as follows: (experimental release − spontaneous release)/(maximum release − spontaneous release). Spontaneous release was measured by incubating target cells alone in medium. Maximum release was measured by lysing target cells with 2% SDS.

Reconstitution by adoptive transfer

Splenocytes (1 × 10⁷/mouse) were injected i.v. into Rag2–/– mice. The next day, both cyclosporine treatment and P815 challenge were initiated.

Results

Proliferation by Ppia−/− cells is cyclosporine resistant

To test whether Ppia−/− cells have altered sensitivity to cyclosporine, the effects of different doses of drug on splenocyte proliferation induced by plate-bound anti-CD3 were quantified (Fig. 1A). Cyclosporine inhibited proliferation of Ppia+/+ or Ppia−/− splenocytes to a similar degree, with an IC₅₀ of ∼25 nM and complete inhibition at ∼100 nM. In striking contrast, the IC₅₀ of Ppia−/− splenocytes was at least 10-fold higher, and complete inhibition was not observed using drug doses up to 2.5 μM. Similar cyclosporine resistance was seen when Ppia−/− splenocytes were stimulated with either Con A or PMA and ionomycin (data not shown). Analysis of purified CD4+ T cells stimulated with anti-CD3 showed that the IC₅₀ of cyclosporine for Ppia−/− cells was also at least 10-fold greater than that of Ppia+/+ cells (Fig. 1B), demonstrating that cyclosporine resistance was intrinsic to T cells. Similar results were obtained when purified CD4+ T cells were stimulated with anti-CD3 in combination with anti-CD28 (data not shown).

As seen with splenocytes, proliferation by Ppia−/− CD4+ T cells was not completely inhibited by any dose of cyclosporine tested, but was diminished at drug concentrations of 250 nM or greater. One explanation for this effect is that another cyclophilin can mediate calcineurin inhibition, but only at high doses of drug. Alternatively, some calcineurin-independent pathway might be affected under these conditions. To distinguish between these possibilities, CD4+ T cell proliferation in the presence of methyl-Ile⁴-cyclosporine was analyzed (Fig. 1C). This cyclosporine derivative has higher affinity for CypA than the parent compound, but completely lacks immunosuppressive activity (33). Methyl-Ile⁴-cyclosporine had no effect on the proliferation of Ppia−/+/ or Ppia−/− CD4+ T cells at doses equivalent to those at which cyclosporine caused inhibition. This result indicates that Ppia−/− cells are partially sensitive to high-dose cyclosporine due to calcineurin inhibition mediated by another cyclophilin family member.
FK506 is another immunosuppressive compound used to prevent organ transplant rejection. Structurally unrelated to cyclosporine, FK506 also binds to and inhibits calcineurin, but only as part of a complex with members of the FKBP family of PPIases (34). Ppia−/− and Ppia+/+ CD4+ T cells stimulated with anti-CD3 had similar responses to different doses of FK506 (Fig. 1D). These results show that proliferation by Ppia−/− cells is dependent on calcineurin activity, indicating that cyclosporine resistance is due to abrogation of calcineurin inhibition rather than gross dysregulation of TCR-induced signaling pathways.

Proliferation by Ppia−/− and Ppia+/+ CD4+ T cells in response to syngeneic and allogeneic BMDDCs was also assessed. The responses of Ppia−/− cells to either syngeneic or allogeneic BMDDCs were slightly lower than those of Ppia+/+ cells (Fig. 2A). To measure cyclosporine responses, CD4+ T cells were mixed with either Ppia+/+ or Ppia−/− allogeneic BMDDCs in the presence of different concentrations of drug (Fig. 2B). Ppia+/+ CD4+ T cells were inhibited to the same extent by cyclosporine regardless of the genotype of the stimulator cell population. Ppia−/− CD4+ T cells showed resistance to cyclosporine when challenged with Ppia+/+ allogeneic BMDDCs, and this was increased when Ppia−/− cells were used as stimulators. These results suggest that cyclosporine, via interactions with CypA, can inhibit the function of APCs.

One well-characterized response to calcineurin activation is induction of IL-2 expression (35). Ppia+/+ and Ppia−/− splenocytes were stimulated with anti-CD3, and accumulation of IL-2 in culture supernatant was determined (Fig. 3A). When no cyclosporine was added, Ppia+/+ and Ppia−/− cells produced similar amounts of IL-2. At low doses of cyclosporine (25 nM), IL-2 production by Ppia+/+ cells was reduced >10-fold, and was undetectable at higher concentrations of drug. In contrast, IL-2 production by

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** The responses of Ppia−/− CD4+ T cells to allogeneic dendritic cells are cyclosporine resistant. A, Proliferative responses of Ppia+/+ and Ppia−/− CD4+ T cells mixed with irradiated, syngeneic or allogeneic Ppia+/+ BMDDCs. B, Proliferative responses of Ppia+/+ and Ppia−/− CD4+ T cells mixed with irradiated, allogeneic Ppia+/+ or Ppia−/− BMDDCs (DCs) in the presence of the indicated concentrations of cyclosporine (CsA). Relative proliferation corresponds to [3H]thymidine incorporation observed relative to that by the control (an identical mixture of CD4+ T cells and DCs lacking cyclosporine).
Ppia−/− cells was modestly decreased by the presence of 25 nM cyclosporine, and remained detectable at drug concentrations up to 750 nM. These results were confirmed by RT-PCR analysis of IL-2 mRNA (Fig. 3B). In the absence of drug, steady-state levels of IL-2 mRNA were similar in Ppia−/− and Ppia+/− cells. Addition of 25 nM cyclosporine greatly reduced IL-2 mRNA in Ppia+/− cells, but had only a slight effect on IL-2 mRNA levels in Ppia−/− cells. Higher concentrations of cyclosporine caused gradual reductions in the amounts of IL-2 mRNA detected in Ppia−/− cells, but the levels seen were still well above those in Ppia+/− cells at drug concentrations up to 250 nM.

Two other genes that are regulated by calcineurin encode the IL-2R α-chain (CD25) and the very early activation Ag CD69 (18). Cell surface expression of these proteins on CD4+ T cells stimulated with anti-CD3 was therefore assessed (Fig. 3C). When no cyclosporine was present, Ppia−/− and Ppia+/− cells expressed similar levels of CD25 and CD69. Addition of 25 nM cyclosporine was sufficient to completely block expression of both proteins on Ppia+/− cells, but caused only a slight decrease in the percentage of Ppia−/− CD4+ T cells expressing high level CD25 and CD69. Increasing doses of drug further reduced the proportion of Ppia−/− cells expressing both markers, but a population staining positive for CD25 and CD69 remained detectable at cyclosporine concentrations up to 250 nM. As expected, doses of FK506 sufficient to inhibit proliferation of CD4+ T cells (Fig. 1D) completely blocked expression of CD25 and CD69 on both Ppia−/− and Ppia+/− cells. These results show that the expression of calcineurin-regulated target genes by Ppia−/− cells is cyclosporine resistant.

Calcineurin and MAPK pathways in Ppia−/− cells are resistant to cyclosporine

In response to increased intracellular calcium levels, calcineurin dephosphorylates the cytoplasmic forms of the NF-ATs, a modification that is blocked by cyclosporine (18). Immunoblot analysis of lysates prepared from lymph node cells stimulated with ionomycin in the absence of cyclosporine showed that the extent of NFATc2/p1 dephosphorylation was similar in Ppia−/− and Ppia+/− cells (Fig. 3D). Pretreatment with 25 nM cyclosporine completely abolished NFATc2/p1 dephosphorylation in Ppia+/− cells treated with ionomycin. In contrast, dephosphorylation of NFATc2/p1 could be detected in ionomycin-stimulated Ppia−/− cells pretreated with concentrations of cyclosporine as high as 750 nM. Thus, based on direct analysis of a physiologic substrate, calcineurin activity in Ppia−/− cells is cyclosporine resistant.

Cyclosporine can suppress activation of p38 MAPK in T cells via inhibition of calcineurin-independent pathways (36). Whether CypA is required for this effect was therefore assessed (Fig. 3E). In the absence of cyclosporine, PMA and ionomycin induced similar levels of p38 phosphorylation in Ppia−/− and Ppia+/− CD4+ T cells. Addition of 40 nM cyclosporine completely inhibited phosphorylation of p38 in Ppia−/− cells, but had no effect on the level of p38 phosphorylation in Ppia+/− cells. Higher amounts of cyclosporine (200 nM) suppressed p38 phosphorylation in Ppia−/− cells (data not shown), suggesting that another cyclophilin can substitute for CypA, but only at high doses of drug. These results show that CypA mediates inhibition of p38 MAPK activation by cyclosporine.

Ppia−/− mice are resistant to immunosuppression by cyclosporine

To test whether CypA is required for the effects of cyclosporine in vivo, H2b recipient mice were challenged with allogeneic H2d P815 mastocytoma cells delivered by i.p. injection. Tumor cell clearance was monitored by flow cytometric analysis of peritoneal exudate cells (Fig. 4A). In the absence of cyclosporine treatment, Ppia−/− and Ppia+/− mice eradicated the tumor cells with similar kinetics (Fig. 4B). Ppia−/− mice treated with 30 mg/kg/day cyclosporine were unable to clear the tumor cells, which expanded and completely overtook the endogenous cell population by day 10 after injection (Fig. 4, C and D). In striking contrast, Ppia+/− mice receiving the same dose of cyclosporine cleared the tumor cells as efficiently as animals given PBS instead of drug.

Cyclosporine resistance in Ppia−/− mice correlates with preservation of T cell responses

Eradication of P815 tumor cells as an allogeneic challenge requires Ag-specific CD8+ T cell responses (37). Robust, specific killing by CD8+ T cells from Ppia+/− and Ppia−/− mice primed with P815 cells was observed (Fig. 5A). The cytotoxic activity of cells from Ppia−/− mice was consistently lower activity than those from Ppia+/− mice, suggesting that CD8+ T cell responses in Ppia−/− mice are slightly impaired. CD8+ T cells from Ppia+/− mice treated with cyclosporine completely lacked killing activity, but
cells from \textit{Ppia}^{-/-} mice given the same dose of drug were potently and specifically cytotoxic (Fig. 5B). Relative to \textit{Ppia}^{+/+} mice given PBS, the specific killing activity generated in cyclosporine-treated \textit{Ppia}^{-/-} mice was greater, indicating that cyclosporine can paradoxically augment CD8^+ T cell responses in these animals.

The cell surface marker CD62L is down-regulated when naive T cells are stimulated (38). Low level expression of CD62L is maintained on effector and memory T cell populations and correlates with cytolytic activity by CD8^+ T cells (39). Following injection of tumor cells, CD62L expression was decreased on CD4^+ cells and CD8^+ cells from the spleens of \textit{Ppia}^{+/+} and \textit{Ppia}^{-/-} mice to a similar extent (Fig. 5C). Down-regulation of CD62L on CD4^+ T cells most likely reflects a specific response to the injected cells, because Th cell can accelerate the clearance of P815 cells (37, 40). When 30 mg/kg/day cyclosporine was administered during the tumor cell challenge, CD62L expression remained high on either CD4^+ or CD8^+ cells from \textit{Ppia}^{+/+} mice, but was low on both cell types from \textit{Ppia}^{-/-} mice. These results indicate that T cell responses to allograft challenge in \textit{Ppia}^{-/-} mice are resistant to cyclosporine.

\textbf{Assessment of the magnitude of cyclosporine resistance in \textit{Ppia}^{-/-} mice}

To roughly determine the difference in cyclosporine sensitivity between \textit{Ppia}^{+/+} and \textit{Ppia}^{-/-} mice, tumor cell clearance in animals treated with different doses of cyclosporine was assessed (Fig. 6A). A dose of 30 mg/kg/day was sufficient to block tumor cell clearance in \textit{Ppia}^{+/+} mice, while administration of 70 mg/kg/day combined with the tumor cell challenge led to premature sacrifice, with evidence that the injected tumor cells had infiltrated the liver (data not shown). In contrast, cyclosporine doses of at least 70 mg/kg/day were required to inhibit tumor cell clearance in \textit{Ppia}^{-/-} mice, which were able to tolerate drug doses up to 90 mg/kg/day and showed no overt signs of tumor growth in the liver.

The killing activity of CD8^+ T cells from \textit{Ppia}^{-/-} mice treated with different doses of cyclosporine was also analyzed (Fig. 6B). Cells from mice given 50 mg/kg/day cyclosporine were potently cytotoxic, while those from animals treated with higher doses of drug had reduced activity. Still, the killing activity by CD8^+ T cells from \textit{Ppia}^{-/-} mice receiving the 90 mg/kg/day drug was greater than that by cells from \textit{Ppia}^{+/+} mice given 3 times less drug, which had essentially no activity.

\textbf{Cyclosporine resistance is transferred with \textit{Ppia}^{-/-} splenocytes}

Two mechanisms could explain the cyclosporine resistance of \textit{Ppia}^{-/-} mice. Cyclosporine might be unable to inhibit immune responses because the necessary receptor is not expressed in target cells. Alternatively, cyclosporine resistance might be secondary to abnormal metabolism of the drug due to the absence of CypA in other nonimmune tissues. To distinguish these possibilities, the effects of cyclosporine on Rag2^{-/-} mice reconstituted with \textit{Ppia}^{+/+} or \textit{Ppia}^{-/-} splenocytes were evaluated. \textit{Ppia}^{+/+} and \textit{Ppia}^{-/-} splenocytes reconstituted the immune system of Rag2^{-/-} recipients with similar efficiencies (Fig. 7A). In the absence of cyclosporine treatment, mice reconstituted with either \textit{Ppia}^{+/+} or \textit{Ppia}^{-/-} cells eradicated the tumor cells with similar efficiency (Fig. 7B). When mice were treated with cyclosporine, the tumor cells expanded in mice reconstituted with \textit{Ppia}^{+/+} cells, but were cleared in those that received \textit{Ppia}^{-/-} cells. These results demonstrate that the lack of CypA expression in immune cells results in cyclosporine resistance.
Discussion

Our results demonstrate that, despite the fact that mammalian cells express multiple cyclophilins with demonstrated or predicted affinity for cyclosporine, CypA is the predominant, if not the sole, mediator of the drug’s immunosuppressive effects. Although cells and mice lacking Ppia remain partially sensitive to high doses of cyclosporine, the concentrations of drug required for these effects are well above that required to bring about immunosuppression in the wild-type counterparts. Our findings also confirm that the molecular basis for immunosuppression by cyclosporine is the formation of a drug-CypA complex that inhibits calcineurin.

Determination of the crystal and solution structure of CypA bound to cyclosporine has identified 15 aa within CypA that mediate contacts with the drug (15, 20). Alignment of the 15 cyclophilins encoded in the mouse genome (Table I) reveals that CypB, C, and F share identity with CypA at all of these residues, while 4 other cyclophilins, CypD, E, Ppi1l, and Riken clone J08, contain single substitutions. CypA, B, and C all bind with high affinity to cyclosporine in vitro (30), whereas secreted CypB augments cellular uptake of cyclosporine via interaction with cell surface binding sites (41). These observations argue that multiple cyclophilins have the potential to interact with cyclosporine in cells. From our studies, we cannot rule out the possibility that ligands other than CypA contribute to the immunosuppressive effects of cyclosporine. Nevertheless, our results clearly demonstrate that CypA is required for immunosuppression by cyclosporine at physiologically relevant concentrations of the drug.

It is widely accepted that a complex formed between cyclosporine and cyclophilins causes immunosuppression by binding to and inhibiting calcineurin (34). Studies in yeast of the effects of cyclosporine on calcineurin-dependent pathways have provided strong support for this model (22, 28, 29, 42). Our data show a strong correlation between the maintenance of calcineurin activity and cyclosporine resistance in lymphocytes.

CypA, B, and C all form complexes with cyclosporine that inhibit calcineurin activity in vitro (30, 43). Moreover, CypB is significantly more efficient than CypA at mediating calcineurin inhibition in vitro (21). The unique importance of CypA for calcineurin inhibition demonstrated in this study must therefore reflect factors other than affinity for cyclosporine or calcineurin. CypA is found at high concentrations in cells, and a pool of CypA must be accessible for interaction with both cyclosporine and calcineurin. CypB is localized to membrane components of the secretory pathway and may be sequestered due to spatial distribution or competing interactions with other cellular factors. An analogous situation seems to apply to the FKBP family of PPIases and the calcineurin inhibitor FK506. Although at least three FKBPs can form complexes with FK506 that inhibit calcineurin, studies using mutant mice have shown that FKBP12 is the sole mediator of the drug’s effect on T cells in vitro (44).

Our data clearly show that CypA is the predominant cyclosporine receptor. Yet, cells lacking CypA are not completely resistant to the drug, because high levels of cyclosporine can inhibit T cell
responses. Although likely to be nonphysiologic, these effects suggest that another cyclophilin can mediate calcineurin inhibition when high concentrations of cyclosporine are present. Previous studies demonstrated that either CypA or CypB is capable of mediating cyclosporine inhibition of calcineurin when overexpressed in a transformed human T cell line (30). However, because CypB is sequestered in the secretory pathway, it is difficult to envision how it might interact with calcineurin. One possible explanation is that high concentrations of CypA disrupt the transport of CypB into the secretory system so that more of this protein is accessible for calcineurin binding. Support for this idea comes from studies showing that cyclosporine alters the trafficking of CypB (45).

Previous studies have indicated that cyclosporine can inhibit p38 MAPK activation via what appears to be a calcineurin-independent pathway (36). In contrast, a more recent report shows that FK506, but not cyclosporine, can inhibit p38 activation in mammalian cells subjected to osmotic stress (46). The fact that both cyclosporine and FK506 have been observed to inhibit p38 supports the hypothesis that this effect involves calcineurin, despite evidence to the contrary. In contrast, p38 MAPK activation appears independent of calcium in transformed B cells (53), which is consistent with the possibility that both FK506 and cyclosporine affect p38 activity by a mechanism that does not involve calcineurin. Our experiments demonstrate that CypA mediates inhibition of p38 MAPK by cyclosporine in T cells, but do not resolve the question of how this comes about.

Although the molecular mechanism by which cyclosporine causes immunosuppression has been studied in detail, the cell types and factors affected by the drug have not been completely defined. Our analysis shows that effective T cell responses coincide with cyclosporine resistance, indicating that T cells are major targets for the drug. Previous studies have established that cyclosporine inhibits expression of multiple cytokines and cell surface markers by T cells, most or all of which are regulated by the NF-AT proteins (18). Mice deficient for both IL-2 and IL-4, or IL-2 and IFN-γ, which are all cyclosporine-sensitive genes, are still capable of rejecting allografts (47, 48). These findings, and the demonstration that allograft rejection in these mutant animals can be delayed by immunosuppressive agents, imply that cyclosporine must affect additional factors to suppress graft rejection.

One such target might be CD154 (CD40L), the molecule of which disruptions interact between T cells and APCs and compromises responses to alloantigens (49, 50). In addition to showing that APC function is critical for allograft rejection, this finding puts forth the possibility that APC may also serve as targets for cyclosporine. Our data showing that the ability of allogeneic dendritic cells to drive CD4+ T cell proliferation is affected by cyclosporine in a CypA-dependent manner support this idea, as do reports suggesting that dendritic cell development and mature cell function are inhibited by the drug (51, 52). The cyclosporine resistance of Ptpa<sup>-/-</sup> mice provides a tool to further test this possibility and, more generally, gain a better understanding of the molecular pathways that must be targeted to suppress allograft rejection. Ptpa<sup>-/-</sup> mice also provide a means to determine whether calcineurin inhibition mediated by CypA is the cause of adverse side effects associated with cyclosporine therapy.

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Disclosures

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