Chapter 5

Pharmacodynamic monitoring of calcineurin inhibition therapy; principles, performance, and perspectives

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Therapeutic Drug Monitoring 2010, 21(1), 3-8
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Abstract

Background: The calcineurin inhibitors cyclosporin A and tacrolimus are immunosuppressive drugs used extensively in allograft recipients. These drugs show large inter-individual pharmacokinetic variation and are associated with severe adverse affects, including nephrotoxicity and cardiovascular disease. In current practice, calcineurin inhibitors are combined with other immunosuppressive drugs such as steroids and mycophenolate mofetil. Dosage is titrated based on blood concentration measurement. For further optimization of calcineurin inhibition therapy, new monitoring strategies are required. Pharmacodynamic monitoring strategies constitute novel approaches for optimization of calcineurin inhibitors therapy.

Content: This review focuses on the general aspects of immunosuppressive drug pharmacodynamic monitoring and describes the methodologies used for monitoring calcineurin inhibition therapy. Two different types of pharmacodynamic monitoring strategies can be distinguished: 1) enzymatic strategies, which monitor inhibition of drug-target enzyme activity, and 2) immunological strategies, which measure cellular responsiveness after in vitro simulated immunological responses. Enzymatic tests are drug type specific markers, in which calcineurin activity is directly determined. Immunological strategies measure immune responsiveness at several levels, such as mRNA transcripts, (intracellular) concentrations/excretion of cytokines, expression of surface activation markers, and cell proliferation. This review also discusses analytical issues and clinical experience with these techniques.

Summary: The call for new methodologies to evaluate immunosuppressive therapy has led to the development of a large variety of pharmacodynamic monitoring strategies. The first reports of their clinical relevance are available, but further understanding of the analytical and clinical variables involved are required for the development of accurate, reproducible, and clinically relevant markers.
Introduction

Organ transplantation is a well-accepted treatment for end-stage organ failure of various organs including the kidney, heart, lung, pancreas, and liver. For successful treatment, adequate suppression of the anti-allograft immunologic response is crucial. For this purpose, a large assortment of small-molecule immunosuppressants is available and used at various stages of the immunosuppressive regimen. These drugs include the calcineurin inhibitors cyclosporine and tacrolimus, the inosine monophosphate dehydrogenase (IMPDH) inhibitor mycophenolic acid, and the mammalian target of rapamycin (mTOR) inhibitors sirolimus and everolimus. Each of these agents act by blocking intracellular lymphocyte response pathways (figure 5.1).

**Figure 5.1. T cell activation in relation to pharmacodynamic monitoring strategies for calcineurin inhibitors.** T cell activation is primed by T cell receptor (TCR) recognition of antigen (Ag) presented by MHC molecules on antigen presenting cells (Signal 1). For activation of T cells, co-stimulatory signals are required (Signal 2). Thereafter, several transcription factors are activated, including NFAT. Nuclear translocation of NFAT is controlled by calcineurin phosphatase, which is inhibited by the calcineurin inhibitors CsA and TRL. NFAT is involved in the transcription of several genes to mRNA transcripts encoding immune regulatory proteins that are presented on the T cell surface. These products include CD40L, Fas, OX40, and the IL-2 receptor α (IL2Rα) and cytokines such as IL-2, IFN-γ, TNFα, and GM-CSF. The interaction between IL-2 and the high affinity IL2Rα induces T cell proliferation (Signal 3). The mTOR inhibitors sirolimus and everolimus inhibit T cell proliferation by inhibition of mTOR downstream of the IL2R. Mycophenolic acid (MPA) inhibits T cell proliferation by inhibiting IMPDH dependant nucleotide synthesis. Calcineurin activity, mRNA cytokine transcripts, cytokines, expression of surface markers, and cell proliferation are used as pharmacodynamic markers for calcineurin inhibition therapy. Adapted from (1).
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There is large inter-patient variation in the pharmacokinetic profile of calcineurin inhibitors. These agents also produce various adverse effects, such as nephrotoxicity and cardiovascular disease(2). In order to control drug over- or under-exposure, therapeutic drug monitoring (TDM) is applied. TDM is performed via determination of whole blood drug concentrations at various time points after drug intake (C0, C2) and estimation of area under the curve (AUC) values(2). These TDM strategies have led to significant improvement in immunosuppressive treatment by reducing episodes of rejection(2). Unfortunately, over 10% of renal transplant recipients experience at least one acute rejection within the first 6 months post-transplant, and drug toxicity and oversuppression of immune function by these drugs seriously hamper long-term success(2,3). A search for new, successful immunosuppressive drugs is ongoing. Until new agents are available, optimization of current treatments is probably the most effective means to improve immunosuppressive treatment(4). Improved monitoring strategies for these drugs, including the integration of pharmacogenetics or/and pharmacodynamics within the current TDM protocols, could further individualize drug dosing and improve clinical performance of these agents(4). Currently, the pharmacogenetic profile of calcineurin inhibitors is used as an extension of pharmacokinetics to predict individual pharmacokinetic profiles and allow for more rapid and simple achievement of therapeutic target drug levels. Pharmacodynamic monitoring strategies measure drug effectiveness parallel to pharmacokinetics, and could thus provide additional information to define new therapeutic target conditions.

General aspects of pharmacodynamic monitoring strategies for calcineurin inhibitors

Why pharmacodynamic monitoring?
The current TDM approaches are based on concentration measurement, and are surrogate markers of drug effect. They are limited in their ability to measure drug effectiveness at its immunosuppressive site of action. Pharmacodynamic strategies are designed to address this issue. A large amount of evidence supports the concept that T cell suppression is the key mechanism by which calcineurin inhibitors achieve immune suppression. T cells are therefore interesting candidates for pharmacodynamic monitoring strategies(5). Current TDM strategies determine blood drug concentrations in extra-cellular or whole blood fractions. However, measurement of whole blood or extra-cellular blood concentrations does not
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necessarily provide an accurate and reproducible reflection of intra-cellular drug concentrations within T cells. For instance, calcineurin inhibitors in whole blood are highly bound to erythrocytes and lipoproteins. Inter- and intra-patient variations of these parameters have been shown to affect calcineurin inhibitor concentrations in lymphocytes(6,7). Another variable that could significantly affect distribution of immunosuppressive drugs in blood are the drug-efflux transporters. Most immunosuppressive drugs are substrates for one or more drug transporter proteins, such as P-glycoprotein, that are expressed on T cells(8,9). Intracellular drug concentrations can therefore be affected by cellular drug-exporting activity. Pharmacokinetic profiles and drug-drug interactions at this level are not monitored by current TDM strategies. In order to evaluate and adjust drug dosage based on these variables, intracellular T cell drug concentration assays have been developed(10).

Drug concentrations are used as a surrogate marker of effect, but do not necessarily correlate with pharmacological response(11). Effect itself is much more complex and may involve drug-independent mediators, such as the requirement of immunophilins for activity of calcineurin inhibitors and mTOR inhibitors. An advantage of pharmacodynamic monitoring strategies is that pharmacological and immunological drug-drug interactions can be detected(12).

Enzymatic versus immunological monitoring strategies

There are two different approaches applied in pharmacodynamic monitoring of immunosuppressive drugs: 1) enzymatic monitoring by measurement of the drug (main) target enzyme, and 2) immunological monitoring by measurement of ex vivo simulated immunological responses. In enzymatic protocols, the intracellular enzyme activity of calcineurin is used to monitor CsA and TRL. This enzyme system is selectively targeted by one class of drugs and will therefore monitor the pharmacodynamics of one immunosuppressive agent (figure 5.2)(13).

In immunological monitoring protocols, stimulated full blood or isolated cell fractions are monitored for T cell activation markers such as cytokine mRNA transcripts, cytokine excretion, and surface activation proteins(12). These responses are more general measures of immune function. Drug pharmacodynamics are determined by quantifying the degree of inhibition of these immunological responses that can be ascribed to the immunosuppressive agents(s)(14). The calcineurin inhibitors are thought to be the major effectors of these parameters, since they are the first in line to interfere with immune functioning(1) and the immunological markers used are primarily controlled by calcineurin(15). Using these strategies, immunosuppressive synergism, additivity,
or antagonism by combinations of various classes of immunosuppressive agents can be detected(12). As a consequence, it is more difficult to ascribe the immune inhibitory effect to a single component of combination drug therapy.

**Figure 5.2. Drug specificity of enzymatic vs. immunological strategies.** IL-2 excretion inhibition after mitogen stimulation (left) and calcineurin inhibition (right) of CsA, TRL, sirolimus (SRL), and everolimus (EVL) in PBMCs isolated from healthy volunteers. Data represent mean of duplicate and are expressed as percent of blank measurement. Adapted from(13).

**Pharmacodynamic monitoring of calcineurin inhibition therapy**

**Pharmacology of calcineurin inhibitors in T cells**
Calcineurin is the Ca\(^{2+}\)-and calmodulin-dependent serine-threonine phosphatase(16). It consists of a regulatory subunit (CNB) and a catalytic subunit (CNA) that becomes available for substrate dephosphorylation after formation of a complex between CNB, calmodulin, and Ca\(^{2+}\)(16). Calcineurin inhibitors inhibit calcineurin after binding an intracellular protein (immunophilin). These complexes block the active site for large substrates(17). There are two different classes of immunophilins: 1) cyclophilins, targeted by CsA, and 2) FK506 binding proteins, targeted by TRL(18).

Calcineurin is involved in a variety of homeostatic processes. Its actions include dephosphorylation of multiple phosphoproteins and phosphopeptides, but calcineurin is probably best known for its role in T cell activation. T cell receptor binding results in increased intracellular Ca\(^{2+}\). After binding of calmodulin, this intracellular Ca\(^{2+}\) activates calcineurin phosphatase activity. Activated calcineurin dephosphorylates NFAT (nuclear factor of activated T cells) transcription complex, which then translocates to the nucleus(19). Upon co-stimulation, NFAT proteins cooperate with members of the activator protein 1 (AP-1) family of transcription.
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factors to transcribe activation-induced genes(20). One of these genes is interleukin 2 (IL-2) whose transcription and excretion is controlled by calcineurin. IL-2 activates various cells of the immune system including helper T-cells, cytotoxic T-cells, NK cells, B cells, and macrophages(21). Other gene products in activated mononuclear cells that are under the control of calcineurin are interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α), and granulocyte-macrophage-colony stimulating factor (GM-CSF)(15,22). IFN-γ is a cytokine predominately produced by activated T and NK cell; it exerts its immuno-modulatory effect by induction of MHC expression(23). The hematopoietic growth factor GM-CSF is produced by a variety of cell types including activated T cells. GM-CSF recruits circulating neutrophils, monocytes, and lymphocytes(24). TNF-α is produced by activated T-cells and macrophages, activates macrophages and lymphocytes, and enhances their cytotoxic potential(25). In addition, transcription of several Th2 cytokines is controlled by calcineurin(15). Among these, IL-4 has a broad range of immune functions, including stimulation of IgE synthesis in B cells.

Calcineurin also controls the expression of T cell surface receptors. Their appearance is associated with T cell activation, and the receptors constitute important immune-response control systems after T cell activation(15). The biological effects of IL-2 are mediated via the IL-2 surface receptors, which is composed of three subunits; the α-chain (CD25), the β-chain (CD122), and the γ-chain (CD132)(21). In normal circulating lymphocytes, the IL-2 receptor α is expressed in less than 5% of cells at very low levels, but is rapidly transcribed and expressed on activated T-cells; this expression is inhibited by CNIs(15,21,26). CD40 ligand (CD40L/CD154) is another surface receptor whose expression is controlled by calcineurin(15,27). Like TNF-α, CD40L is a member of a large family of structurally related ligands that are referred to as the TNF superfamily. These proteins regulate functions such as immune responses and haematopoiesis(28). CD40L is selectively expressed on activated mature T cells and not on resting T cells(29). Its surface expression is controlled by translocation of preformed CD40L and mRNA-mediated CD40L expression after anti-CD3 stimulation. This expression is observed as early as 1-2 hours after stimulation and peaks at 6 to 8 hours(29). The effects of TNF ligands are mediated via TNF receptors. Several of these ligands are expressed on T cells after activation, and act to control duration of T cell responses and T cell survival. One of these ligands is OX40 (CD134), which is crucial for T-cell activation and survival(30). OX40 expression is induced by T cell receptor stimulation. Expression peaks around 2 to 3 days after stimulation and is lost 4 to 5 days after stimulation(31). Another TNF receptor that is selectively expressed on activated T cells is Fas (CD95). Interaction of Fas with
its ligand (FasL/CD178) triggers rapid apoptosis of activated T cells(32). Other T cell receptors that show expression on activated T cells are involved in cell adhesive interactions. The leukocyte function associated antigen (LFA-1/CD11a) is activated upon TCR stimulation, resulting in increased affinity for its counter-receptors. This interaction is important for T cell adhesion to endothelial cells, and for diapedesis through endothelium. It also acts as a co-stimulatory signal in T cell interactions(33). Two other surface activation receptors are CD69 and CD71. Activation induced molecule (AIM/CD69) is present on activated but not resting T cells, and appears relatively early after T cell activation. CD69 is important in cell activation, proliferation, and differentiation(34). The transferrin receptor (TfR/CD71) binds iron-loaded transferrin and plays a key role in cell proliferation control, since iron is a key nutrient required for cell proliferation. TfR constitutively cycles between the endosomal compartment and plasma membrane. Increases in T cell surface expression are observed after T cell stimulation(35).

Calcineurin activity measurement
Calcineurin enzyme activity is determined in excess of all required reagents. Therefore in vitro enzyme activity does not necessarily reflect the actual intracellular enzyme activity. In this context, this technique may be more correctly referred to as a capacity measurement that reflects binding of calcineurin inhibitor/immunophilin complexes to calcineurin when fully active(5). Calcineurin enzyme activity is regulated intracellularly by Ca\(^{2+}\); in naïve T cells, no sustained elevation of calcium is present. From an immunological point of view, a lack of sustained intracellular hypercalcemia means there is no activation of T cells via nuclear factor of activated T-cells (NFAT); thus no suppression of the calcineurin pathway is theoretically required. T cells activated by T-cell receptor binding of allo-antigen have sustained elevation in Ca\(^{2+}\) concentrations and calcineurin activity. Theoretically, only these cells would require calcineurin inhibition in order to suppress the immune response. Adequate binding to and inhibition of calcineurin in these cells is therefore crucial for successful immunosuppression. In vitro assays approximate these intracellular conditions.

In the calcineurin enzyme activity protocols, the Ser-phosphorylated 19-amino acid peptide Asp-Leu-Asp-Val-Prol-Ile-Pro-Gly-Arg-Phe-Asp-Phe-Asp-Arg-Arg-Val-Ser-Val-Cys-Ala-Glu is used as substrate, which corresponds to residues 81-99 of the bovine cardiac cAMP-dependent protein kinase regulatory subunit (R\(_\text{II}\)). This substrate is not specific for calcineurin, and it can be dephosphorylated by other serine-threonine phosphatases (PP)(36). There are four serine-threonine phosphatase enzymes (PP1, PP2A, calcineurin, and PP2C). For selective
calcineurin activity determination, okadaic acid is used as a specific inhibitor of PP1 and PP2A. To distinguish calcineurin from PP2C, calcineurin phosphatase activity measurement is defined as CsA-inhibitable or Ca^{2+} dependant phosphatase activity. In the last case, the Ca^{2+} chelator EGTA is used to bind Ca^{2+}, removing free Ca^{2+} from the assay. Interestingly, when these two different approaches are used in vivo, differences in relative PP2C activity are observed. When CsA is used to define calcineurin activity, PP2C phosphatase activity is much larger in the calcineurin + PP2C fraction (> 60%(37) of total serine-threonine phosphatase activity) than Ca^{2+}/calmodulin defined calcineurin activity (< 5%(38,39)). This difference can be explained by the mechanism of calcineurin inhibition by CsA. CsA itself does not inhibit calcineurin, but requires cyclophilin. Cyclophilins are not abundant, and at high CsA concentrations, they limit the ability of CsA to inhibit calcineurin(40,41).

For quantification of the R_{II} substrate dephosphorylation, several detection techniques are used. These techniques are based on either determination of the radioactive ^{32}P isotope, HPLC-UV detection of dephosphorylated peptide, or absorbance measurement of phosphate reagent. An overview of the calcineurin assay methodologies is shown in table 5.1.

Sample choice and preparation
Another issue that has been thoroughly investigated and discussed is sample choice for calcineurin activity monitoring. The major therapeutic target compartment of calcineurin inhibitors is thought to be the T lymphocyte fraction, since calcineurin inhibition within these cells is thought to be crucial for their immunosuppressive action. Monitoring calcineurin activity in this fraction would therefore be most relevant. Unfortunately, no protocol for selective measurement in this cell fraction has been developed, though recently an assay for IMPDH activity in CD4+ cells has been described(42). Calcineurin activity and inhibition by calcineurin inhibitors is monitored in whole blood, leukocyte, peripheral blood mononuclear cell (PBMC), and lymphocyte fractions. Two important features of a suitable sampling fraction are that the fraction is representative of the immunosuppressive site of action and that it is compositionally stable in the sense that compositional changes should not affect measurement outcome. Large differences in PBMC sample composition are observed for renal transplant recipients early after transplantation that can directly confound calcineurin measurement based on differences in cell-specific calcineurin activities of leukocyte subsets(43).
### A) Calcineurin activity

<table>
<thead>
<tr>
<th>Sample</th>
<th>Detection technique</th>
<th>Definition of calcineurin activity</th>
<th>Refs</th>
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</thead>
<tbody>
<tr>
<td>Whole blood</td>
<td>Phosphate, by released $^{32}$P</td>
<td>Phosphatase activity in the presence of OA ± CsA</td>
<td>11,37</td>
</tr>
<tr>
<td>Whole blood</td>
<td>Phosphate, by released $^{32}$P</td>
<td>Phosphatase activity in the presence of OA ± EGTA</td>
<td>46,66</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>Phosphate, Malachite Green</td>
<td>Phosphatase activity in the presence of OA ± EGTA</td>
<td>39</td>
</tr>
<tr>
<td>PBMC</td>
<td>Phosphate, by released $^{32}$P</td>
<td>Phosphatase activity in the presence of OA ± CsA</td>
<td>11,67</td>
</tr>
<tr>
<td>PBMC</td>
<td>Phosphate, by released $^{32}$P</td>
<td>Phosphatase activity in the presence of OA ± EGTA</td>
<td>38,46</td>
</tr>
<tr>
<td>PBMC</td>
<td>Dephosphorylated RII, HPLC</td>
<td>Phosphatase activity in the presence of OA</td>
<td>51,63</td>
</tr>
<tr>
<td>PBMC</td>
<td>Phosphate, by released $^{32}$P</td>
<td>Phosphatase activity in the presence of OA and Mg</td>
<td>52</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>Dephosphorylated RII, HPLC</td>
<td>Phosphatase activity in the presence of OA</td>
<td>68</td>
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</tbody>
</table>

### B) Cytokine mRNA

<table>
<thead>
<tr>
<th>Sample</th>
<th>Stimulation agents</th>
<th>Cytokines</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes/PBMC</td>
<td>PMA/ionomycin</td>
<td>IL-2, IFN-γ, GM-CSF</td>
<td>60,69</td>
</tr>
<tr>
<td>Leukocytes/PBMC</td>
<td>Anti-CD3/anti-CD28</td>
<td>IL-2, IL-4, TNFα</td>
<td>57,70</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>Anti-CD3/anti-CD28</td>
<td>IL-2</td>
<td>59</td>
</tr>
</tbody>
</table>

### C) Extracellular cytokine concentration

<table>
<thead>
<tr>
<th>Sample</th>
<th>Stimulation agents</th>
<th>Cytokines</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant stimulation matrix</td>
<td>Anti-CD3/anti-CD28</td>
<td>IL-2</td>
<td>59,70</td>
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<tr>
<td>Blood supernatant</td>
<td>PHA</td>
<td>IL-2, IFN-γ</td>
<td>67,71</td>
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</table>

### D) Intracellular cytokine concentration / cytokine producing cells

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Stimulation agents</th>
<th>Cytokines</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ and CD8+ T cells</td>
<td>PMA/ionomycin</td>
<td>IL-2</td>
<td>63</td>
</tr>
<tr>
<td>CD4+ and CD8+ T cells</td>
<td>PMA/ionomycin</td>
<td>IL-2, IFN-γ, TNFα, IL-4</td>
<td>72</td>
</tr>
<tr>
<td>T cells</td>
<td>PMA/ionomycin</td>
<td>IL-2, IFN-γ, TNFα, IL-4</td>
<td>14,62,73</td>
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### E) Surface activation markers

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<th>Markers</th>
<th>References</th>
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<tr>
<td>CD4+ T cells</td>
<td>Anti-CD3/anti-CD28</td>
<td>CD25, CD69</td>
<td>70</td>
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<tr>
<td>T cells</td>
<td>Concanavalin A</td>
<td>CD11a, CD25, CD71, CD95, CD134, CD154</td>
<td>62, 72-74</td>
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</table>

### F) Cell proliferation

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Stimulation agents</th>
<th>Markers</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>Concanavalin A</td>
<td>PCNA</td>
<td>62, 72-74</td>
</tr>
</tbody>
</table>

**Table 5.1. Overview of methods used for pharmacodynamic monitoring of calcineurin inhibitor therapy.** Methods are listed per strategies (A-F) and are subdivide by characteristics. Abbreviations: CsA, cyclosporin A; EGTA, ethyleneglycoltetraacetic acid; IFN-γ, interferon-γ; IL-2, interleukin-2; OA, okadaic acid; PBMC, peripheral blood mononuclear cells; PCNA, proliferating cell nuclear antigen; PHA, phytohemagglutinin; PMA, phorbol 12-mystrate 13 acetate; TNFα, tumor necrosis factor α.
Sample choice can also affect the inhibition profile of calcineurin inhibitors: inhibition affinities for CsA were comparable for leukocyte subsets, but erythrocytes had significant lower CsA affinity\(^{(40)}\).

Another problem that has been highlighted is the problem of calcineurin activity loss due to fractionizing and separation procedures\(^{(5)}\) and washing out of drugs during cell preparation or by drugs efflux transporters. To overcome some of these issues, samples have been treated with inhibitors of drug-efflux transporters or have been prepared on ice\(^{(10,39)}\).

**CN activity in vivo**

Two comprehensive reviews were recently published that report the clinical performance of current assays in detail\(^{(44,45)}\). Here we briefly discuss the general outlines of these studies. When calcineurin activity is monitored in transplantation patients treated with either TRL or CsA, a clear inverse relation between drug concentration and enzyme activity in whole blood, leukocytes, and PBMC fraction is found\(^{(39,46,47)}\). A representative example of calcineurin activity and CsA drug concentration profiles in a renal transplantation patient the first 6 months after transplantation is shown in figure 5.3.

![Figure 5.3. Leukocyte calcineurin activity and blood CsA concentration of representative renal transplantation patient.](https://example.com/fig53.png)

**Figure 5.3. Leukocyte calcineurin activity and blood CsA concentration of representative renal transplantation patient.** Calcineurin activity and CsA blood concentrations were determined at four time points. Pre-transplant, before starting CsA therapy; week 2 and week 6 after transplantation when reference AUC was 5400 \(\mu g/L\cdot h\), and 6 after transplantation when reference AUC was 3250 \(\mu g/L\cdot h\). 1,2,3,4,5, and 6 represent hours after drug intake, 0 being just before drug intake and 12 hours after previous dose. Adapted from\(^{(65)}\).
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The inhibition of calcineurin activity by CsA in these fractions is rapidly reversible, and no time lag is observed between CsA drug concentration and calcineurin activity(46,48). Interestingly, in the first hours after drug intake, clear inhibition of calcineurin activity is observed in patients. After six hours, calcineurin activity cannot be distinguished from pre-intake calcineurin activity, while drug concentration is still significantly elevated(46,47). Population PK/PD analysis of living donor liver transplant recipients showed large inter-individual variability in the IC$_{50}$ value of CsA 200 μg/L (CV = 84%) and TRL 26.4 μg/L (CV = 81%)(38). In addition, TRL shows a less dynamic change when compared to CsA in the PBMC fraction, and the maximal inhibitory effect of TRL is lower, findings which correspond to in vitro data(38). Another characteristic of calcineurin inhibition by either CsA or TRL is that enzyme activity is only partially inhibited in vivo. This partial calcineurin inhibition results in greater inhibition of weak stimulatory signals compared to maximally stimulatory signals, and is thought to be responsible for the presence of immune responsiveness against infections while patients are successfully protected from allograft rejection(49). Immunophilin concentrations limit calcineurin inhibition by CsA and TRL at high drug concentrations in in vitro systems(41). Whether these effects occur under physiological conditions in transplant recipients remains unclear, since TRL and CsA concentration extrapolations are necessary to translate in vitro results to the in vivo situation. This translation is necessary due to abundant irrelevant binding sites in blood that are absent in culture medium(50).

In order to prove that pharmacodynamic monitoring strategies act as a functional index of calcineurin inhibitor-based immune suppression, correlation between clinical immunological events and measurement is required. Sanquer et al. showed that patients with acute grade II or higher graft-versus-host-disease (GVHD) had greater calcineurin activity, which could be predicted with a sensitivity of 89% and a specificity of 54%(51). However, others have found a decrease in calcineurin activity in bone marrow transplanted patients with acute GVHD, suggesting that CsA-resistance could be independent of calcineurin inhibition(52). In liver transplantation patients, Fukudo et al. showed that calcineurin activity was increased in both TRL and CsA treated patients who suffered from an acute rejection(38). In renal transplant recipients, baseline calcineurin enzyme inhibition was significantly lower in patients with biopsy-proven acute rejection(53).
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Immunological monitoring strategies

Immunological monitoring strategies of calcineurin inhibitor therapy were developed early after the introduction of CsA and well before calcineurin was identified as its pharmacological target protein(54). In these assays, third-party mixed lymphocyte reactions and IL-2 excretion after mitogen stimulation were used as immunologic markers for CsA therapy(54,55). Simulation of an immune response, either by mixed lymphocyte reaction, superantigens, or TCR/co-stimulation directed activation is required for immunological calcineurin inhibitor monitoring strategies. Inhibition of immune responsiveness by immunosuppressant therapy is often used as readout. This inhibition is measured on different levels, including cytokine mRNA, activation surface markers, cytokine producing cells, extracellular cytokine concentration, and cell proliferation (see Figure 1 and Table 1). When patients treated with calcineurin inhibitors are monitored, full blood samples or isolated cell fractions are stimulated and incubated for a period of time to allow cellular responses and the appearance of markers. Distinct kinetic profiles are observed for different markers after stimulation. In addition, the type of stimulus employed, affect the kinetic profile of surface markers(56) and cytokine mRNA(57). When mRNA gene expression programs were analyzed by microarrays, highly similar gene expression patterns were observed for CD3/CD28, phytohemagglutinin (PHA), and ionomycin/phorbol-12-myristate-13-acetate (PMA) stimulation protocols, illustrating the overlapping usefulness of different types of stimulation(58).

For analyzes of cytokine mRNA, mRNA is isolated from leukocyte or PBMC fraction. Quantification of cytokine mRNA is performed by quantitative real-time PCR and expressed with respect to housekeeping mRNA (β-actin, cyclophillin B, or GAPDH)(57,59,60). Several cytokine mRNA transcripts are used as pharmacodynamic markers of calcineurin inhibitor therapy, including IL-2, IFNγ, TNFα, IL-4, and GM-CSF. When blood is incubated with a calcineurin inhibitor, a significant reduction in these transcripts is found. In addition, when cytokine transcripts (IL-2, IFNγ, and GM-CSF) were monitored in renal, cardiac, and liver allograft recipients undergoing immunosuppressive therapy, expression was decreased and showed an inverse relationship with CsA concentrations. Maximum inhibition was observed 2 hours after drug intake corresponding to the peak of CsA drug concentration. As observed for calcineurin enzyme inhibition, gene expression of NFAT-regulated genes 6 hours after drug intake was comparable to through levels(60). In addition, NFAT-regulated gene expression of IL-2, IFN-γ, and GM-CSF was found to be lower in renal allograft patients on CsA that suffered from recurrent infections and malignancies(61).
Determination of cytokine producing cells and surface markers is performed using flow-cytometry to analyze stained cells. For intracellular cytokine analyzes, secretion inhibitors are used during stimulation protocols. Next, permeabilized cells are stained and analyzed by flow cytometry and expressed as percent of cytokine producing cells(14,62). When monitoring cytokine producing lymphocytes in liver allograft recipients, it was found that the percent of IL-2 producing CD8+ T cells was higher during acute rejection compared to non rejecting recipients, with a positive predictive value of 58% and a negative predictive value of 98%(63). No difference was found between percentage of IL-2 producing CD4+ T cells and total T cells, suggesting an important role for IL-2 producing CD8+ T cells.

When cell surface markers are monitored, the expression of T-cells activation markers is determined using monoclonal antibodies. Positive cell populations are quantified by flow cytometry and expressed as percent of marker positive cells within the T cell population(14,62). These strategies have primarily been used to study immunosuppressive combination protocols for drug interactions(12). When monitored in heart transplanted patients on a CsA-based immunosuppressive regime, reductions of the proliferation marker PCNA as well as the T cell surface markers CD11a, CD25, CD71, CD95, CD134, CD152, and CD154 were observed 2 hours after drug intake(64).

**Future perspectives**

The current technologies used for pharmacodynamic monitoring of calcineurin inhibition therapy have not proved to be clinically useful markers in the management of patients on these drugs. More specific samples combined with improved understanding and anticipation of analytical variation, particularly concerning biological assays, might be important for the development of more accurate markers. Most pharmacodynamic monitoring techniques used to date have been validated for their analytical performance, but validation methods such as sample composition effects on measurement and drug leakage/dilution during processing are of key importance. For instance, it is required that the *in vivo* conditions are maintained during the cell isolation/preparation protocol in order to measure a representative pharmacodynamic state. It is assumed that pharmacodynamic measurement in blood or blood fractions are representative of the pharmacodynamic effects at the immunological sites responsible for allograft rejection.

An interesting next step would be to monitor calcineurin capacity or immune responses within T cells, which are the specific immunosuppressive target compartment of the calcineurin inhibitors(5). This method should avoid analytical
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variation by sample composition, and absolute values should be more comparable between individuals. These immunosuppressive capacities could provide a threshold under which no functional T cell activation is possible, even in the presence of full T cell receptor binding and maximum intracellular Ca\textsuperscript{2+} influx. When such new methods have been developed, clinical validation by means of large cohorts is crucial to investigate their efficacy and to determine whether pharmacodynamic monitoring strategies are viable for clinical use.

Conclusions

The call for more accurate and clinically useful methods for monitoring immunosuppressive drugs has led to the development of various pharmacodynamic monitoring strategies for calcineurin inhibitors and other immunosuppressive agents. The study of pharmacodynamic monitoring of immunosuppressive therapy is still in its infancy. Its true efficacy remains to be proven, though the first signs of its clinical utility are emerging. Further understanding and anticipation of the analytical and clinical variables seem to be required for the development of new markers for monitoring calcineurin inhibition therapy in allograft recipients.

References

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