

**CENTER FOR DRUG EVALUATION AND  
RESEARCH**

*APPLICATION NUMBER:*

**206406Orig1s000**

**MICROBIOLOGY/VIROLOGY REVIEW(S)**

**MICROBIOLOGY/IMMUNOLOGY REVIEW**  
**DIVISION OF TRANSPLANT AND OPHTHALMOLOGY PRODUCTS**

**NDA # 206406** (SDN-001; Original)      **REVIEWER** : Shukal Bala  
**CORRESPONDENCE DATE** : 12/28/2013  
**CDER RECEIPT DATE** : 12/30/2014  
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**REVIEW COMPLETE DATE** : 07/23/2014

**APPLICANT:** Veloxis Pharmaceuticals, Inc.  
499 Thornall street  
3<sup>rd</sup> floor,  
Edison, NJ 08837

**DRUG CATEGORY:** Immunosuppressive agent

**INDICATION:** Prophylaxis of organ rejection in adult patients receiving kidney transplants

**DOSAGE FORM:** Tablets for oral administration

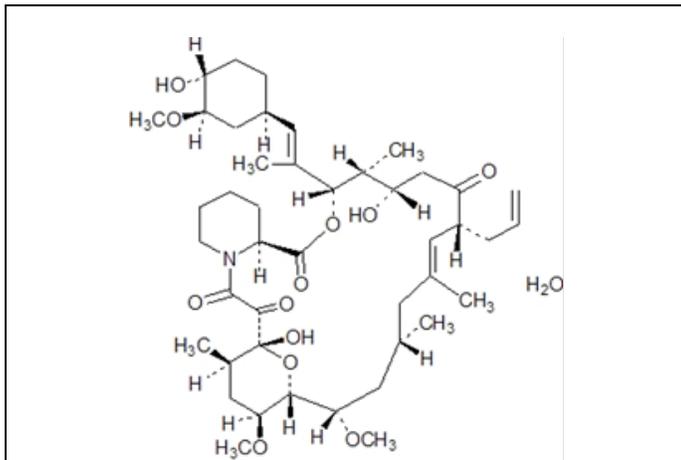
**PRODUCT NAMES:**

a. **PROPRIETARY:** ENVARUSUS-XR

b. **NONPROPRIETARY:** Tacrolimus; FK506

c. **CHEMICAL:** [3*S* – [3*R*\*[*E*(1*S*\*, 3*S*\*, 4*S*\*), 4*S*\*, 5*R*\*, 8*S*\*, 9*E*, 12*R*\*, 14*R*\*, 15*S*\*, 16*R*\*, 18*S*\*, 19*S*\*, 26*aR*\*]] – 5, 6, 8, 11, 12, 13, 14, 15, 16, 17, 18, 19, 24, 25, 26, 26*a* – hexadecahydro – 5, 19 – dihydroxy – 3 – [2 – (4 – hydroxy – 3 – methoxycyclo – hexyl) – 1 – methylethenyl] – 14, 16 – dimethoxy – 4, 10, 12, 18 – tetramethyl – 8 – (2 – propenyl) – 15, 19 – epoxy – 3*H* – pyrido[2, 1 – *c*][1, 4] oxazacyclotricosine – 1, 7, 20, 21(4*H*, 23*H*) – tetrone, monohydrate.

**STRUCTURAL FORMULA:**



Molecular Weight: 822.03

Molecular Formula: C<sub>44</sub>H<sub>69</sub>NO<sub>12</sub>•H<sub>2</sub>O

**SUPPORTING DOCUMENTS:** Prograf<sup>®</sup> labeling

## Table of Contents

<b>1. Introduction and Background .....</b>	<b>3</b>
<b>2. Mechanism of Action .....</b>	<b>3</b>
2.1. Effect on cytokines and chemokines .....	4
• Kino <i>et al.</i> , 1987 .....	4
• Zipperle <i>et al.</i> , 1997 .....	4
• Kohyama <i>et al.</i> , 1999 .....	6
• Lemster <i>et al.</i> , 1995 .....	8
• Jiang <i>et al.</i> , 1999 .....	11
• Khanna <i>et al.</i> , 1999 .....	12
• Sakuma <i>et al.</i> , 2001 .....	15
2.2. Effect on IL-2 receptor expression on lymphocytes .....	16
2.3. Effect on nitric oxide and NF- $\kappa$ B .....	17
• Conde <i>et al.</i> , 1995 .....	17
• Kaibori <i>et al.</i> , 1999 .....	18
2.4. Effect on apoptosis .....	21
<b>3. Conclusions .....</b>	<b>28</b>
<b>4. The Labeling .....</b>	<b>31</b>
4.1. The applicant's version of the labeling .....	31
4.2. FDA's version of the labeling .....	31
<b>5. Recommendations .....</b>	<b>32</b>

## 1. Introduction and Background

The subject of this 505(b)(2) NDA is tacrolimus extended release (ENVARUSUS-XR) tablets, once daily, for the prophylaxis of organ rejection in adult patients receiving kidney transplants. Immediate-release capsules of tacrolimus (Prograf®) are approved in the United States for prophylaxis of organ rejection in patients receiving allogeneic liver, kidney, or heart transplants and require twice daily oral dosing. Prograf® was first approved in 1994 for the treatment of liver transplant.

ENVARUSUS XR, a new extended-release formulation of tacrolimus, was prepared using the MeltDose® drug-delivery technology; according to the applicant this should enhance bioavailability and may reduce the intra-individual variability in tacrolimus absorption.

The applicant refers to the listed drug (LD) Prograf® labeling for section 12.1 entitled “Mechanism of Action” and is same as RLD labeling.

## 2. Mechanism of Action

Tacrolimus is known to inhibit cellular responses; it inhibits allogeneic- and mitogen-induced stimulation of T cell proliferation and inhibits the mixed lymphocyte reaction and generation of cytotoxic T-cells. T cell dependent B cell activation and proliferation is inhibited, and the drug appears to have a direct effect via inhibition of calcium-dependent B cell activation pathways.

The mechanism of section for Prograf® labeling is based on review of studies in 1993 – 1994 (for details see Pharmacology/Toxicology review dated December, 1993; Medical Officer review dated June, 1994) and reads as follows:

Tacrolimus is known to inhibit T-lymphocyte activation by tacrolimus binding to an intracellular protein, FKBP-12. A complex of tacrolimus-FKBP-12, calcium, calmodulin, and calcineurin is then formed and the phosphatase activity of calcineurin inhibited. This effect may prevent the dephosphorylation and translocation of nuclear factor of activated T-cells (NF-AT), a nuclear component thought to initiate gene transcription for the formation of lymphokines such as interleukin-2, gamma interferon. The net result is the inhibition of T-lymphocyte activation (i.e., immunosuppression).

No new nonclinical studies were performed by the applicant; however, some of the publications supporting activity of tacrolimus *in vitro* and/or *in vivo*, included in the NDA submission and obtained by an independent literature search were reviewed. Some of the studies were in various animal models of transplantation and support activity of tacrolimus in improving animal and graft survival. As most of these studies have now been superseded by clinical data, this review focuses only on those studies supporting additional information for mechanism of action than currently described in the Prograf® labeling.

## 2.1. Effect on cytokines and chemokines

- **Kino *et al.*, 1987**

Kino *et al.* (1987)<sup>1</sup> reported the effect of tacrolimus on IL-3 production by murine splenocytes *in vitro*. Briefly, FDC-P2 cells (a murine bone marrow cell line;  $1 \times 10^4$ ), known to be IL-3 dependent, were cultured at 37°C for 24 hours with various dilutions of IL-3 containing supernatants from mixed lymphocyte reaction (MLR) cultures of mouse splenocytes incubated with or without tacrolimus for different time points (0, 24, 48, 72, and 96 hours); cyclosporine A (CsA) was used as a comparator. Supernatant from an IL-3 producing cell (WEHI-3 cell line -  $10^6$ /ml) culture was used as a positive control. Uptake of <sup>3</sup>H thymidine was measured by pulsing cultures with 0.5 µCi of <sup>3</sup>H-thymidine for 4 hours. The unit value was calculated by dilution analysis of test samples and was compared with a laboratory standard preparation in which 100 U was equivalent to the amount of IL-3 necessary to achieve 50% proliferation of FDC-P2 cells. The results showed a decrease in IL-3 production in the supernatants from MLR cultures containing tacrolimus or CsA (Table 1). IC<sub>50</sub> value of tacrolimus (0.3 nM i.e., 246.61 pg/mL) was approximately 10-fold lower than CsA.

Table 1: Effect of FK-506 and CsA on IL-3 production of supernatants from mouse MLR.

Immunosuppressants (nM)	IL-3 production: U/ml (% inhibition)			
	Day 1	Day 2	Day 3	Day 4
Control 0	1.7 (0)	37.0 (0)	41.3 (0)	52.5 (0)
FK-506 10	0 (100)	0 (100)	0.1 (99)	2.6 (95)
1	0 (100)	0.3 (99)	4.6 (89)	8.7 (83)
0.3	0 (100)	10.3 (73)	21.3 (48)	22.6 (57)
0.1	1.1 (35.3)	18.8 (50)	39.7 (4)	35.0 (33)
CS 100	0 (100)	0 (100)	1.8 (96)	10.0 (81)
32	0 (100)	11.1 (71)	44.7 (—)	45.9 (13)
10	1.1 (35)	22.9 (39)	51.9 (—)	45.0 (14)
3.2	0.6 (65)	23.3 (38)	48.0 (—)	72.7 (—)

FK506=tacrolimus; CS = CsA

Comment:

Study showed that tacrolimus decreased IL-3 production.

- **Zipperle *et al.*, 1997**

Zipperle *et al.* (1997)<sup>2</sup> reported the effect of tacrolimus on IL-6 and IL-10 production by B-cell enriched, T-cell enriched, or CD4<sup>+</sup> enriched cells from the peripheral blood mononuclear cells from liver transplant recipients treated with tacrolimus (n=16) or CsA (n=25); cells from 20 healthy controls were included for testing. Of the 41 liver transplant recipients, 26 patients had stable graft function (SGF), seven had impaired graft function (IGF), and eight had ischemic type biliary lesions (ITBL). Cells were stimulated *in vitro* with *Staphylococcus aureus* Cowan (SAC)

<sup>1</sup> Kino T, Hatanaka H, Miyata S, Inamura N, Nishiyama M, Yajima T, Goto T, Okuhara M, Kohsaka M, Aoki H, and Ochiaia T. FK-506, a novel immunosuppressant isolated from a Streptomyces. II. Immunosuppressive effect of FK-506 *in vitro*. J Antibiotics (1987) 9: 1256-1265.

<sup>2</sup> Zipperle S, Weimer FL, Golling M, Daniel V, Otto G, and Opelz G. Impaired T-Cell IL-10 secretion and CD4 helper function in liver transplant patients treated with tacrolimus. Transplant Proc (1997) 29: 1079-1080.

ENVARUSUS-XR

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antigen; patient T-cells, CD4<sup>+</sup> cells, or CD8<sup>+</sup> cells were co-cultured with healthy control B-cells and stimulated with pokeweed mitogen (PWM). Cells were cultured for 6 days and IL-6 and IL-10 measured in the supernatants by enzyme linked immunosorbent assay (ELISA). IL-6 and IL-10 levels were decreased in cells from patients treated with tacrolimus or CsA compared to untreated controls and stimulated with SAC (Table 2). IL-6 and IL-10 levels showed a trend towards decrease in co-cultures of T-cells and its subsets stimulated with PWM; however, the differences were not statistically significant (Table 2). CsA treatment does not appear to be effective in reducing IL-10 production.

Table 2: *In vitro* B Cell response, unstimulated IFN- $\gamma$  response, PWM-stimulated T cell response, and PWM-stimulated CD4 cell response

	Controls	CyA-Treated Patients	P Values*	Tacr-Treated Patients	P Values*
SAC I stimulated B cell response:					
ISC response (ISC/10 <sup>6</sup> B cells)	1187 $\pm$ 439	448 $\pm$ 169	<.02	509 $\pm$ 356	<.02
IL-6 response (pg/mL)	6081 $\pm$ 452	4518 $\pm$ 548	NS	3762 $\pm$ 607	<.01
IL-10 response (pg/mL)	970 $\pm$ 30	588 $\pm$ 91	<.0005	502 $\pm$ 110	<.0005
PWM-stimulated B cell response:					
ISC response (%)	55 $\pm$ 16	52 $\pm$ 19	NS	22 $\pm$ 8	NS
IL-6 response (pg/mL)	97 $\pm$ 15	67 $\pm$ 13	NS	72 $\pm$ 14	NS
IL-10 response (pg/mL)	60 $\pm$ 13	46 $\pm$ 13	NS	52 $\pm$ 17	NS
Unstimulated IFN- $\gamma$ response (pg/mL)	6571 $\pm$ 1025	2539 $\pm$ 840	<.005	2722 $\pm$ 1091	<.01
PWM-stimulated T cell response:					
T cell help (%)	74 $\pm$ 17	66 $\pm$ 16	NS	30 $\pm$ 14	<.02
T cell IL-2 secretion (%)	122 $\pm$ 19	60 $\pm$ 13	<.02	69 $\pm$ 14	NS
T cell IL-10 secretion (%)	90 $\pm$ 11	85 $\pm$ 14	NS	56 $\pm$ 10	<.05
PWM-stimulated CD4 cell response:					
CD4 cell help (%)	109 $\pm$ 18	78 $\pm$ 16	NS	45 $\pm$ 15	<.02
CD4 cell IL-2 secretion (%)	157 $\pm$ 14	86 $\pm$ 15	<.01	100 $\pm$ 21	<.05
CD4 cell IL-10 secretion (%)	129 $\pm$ 26	90 $\pm$ 14	NS	85 $\pm$ 14	NS

Results are given as means  $\pm$  SEM.

\*Wilcoxon's rank-sum test was used for statistical comparison between healthy controls and the respective patient groups (NS = not significant).

Patient B-cells ( $5 \times 10^4$ ) and  $10^5$ T-cells of a healthy control were co-cultured with or without PWM (1%),  $10^5$  patient B-cells were co-cultured with or without SAC I (0.007%). All cultures were performed in duplicate. B-cell responses were assessed in a reverse hemolytic plaque assay and by ELISA determination of IL-6 and IL-10 in culture supernatants.

ISC=immunoglobulin secreting cells

#### Comment:

The study showed that tacrolimus treatment decreased IL-6 and IL-10 production by B-cells cultured and stimulated with SAC I, a T-cell independent antigen, *in vitro*. Similar trend was observed upon stimulation with PWM, a non-specific mitogen a T-cell dependent antigen; however, such inhibition of IL-6 or IL-10 levels was not statistically significant for co-cultures of B-cells or CD4<sup>+</sup> cells. Cytokine levels were low even in co-cultures of cells from healthy control subjects. It is unclear if such differences are due to differences in the different experimental conditions. For example, for PWM stimulated assay patient B-cells or CD4<sup>+</sup> cells were cultured in an allogeneic co-culture system with cells from healthy control subjects in the presence or absence of PWM whereas SAC I stimulated assay was not a co-culture assay. Also, differential stimulatory effect of PWM vs. SAC I on different cell populations cannot be ruled out.

- **Kohyama *et al.*, 1999**

Kohyama *et al.* (1999)<sup>3</sup> reported the *in vitro* effect of tacrolimus on IL-8 produced by eosinophils isolated from the blood of patients who had allergic disease such as mild bronchial asthma and atopic dermatitis; patients had received neither systemic nor inhaled corticosteroid treatment. Eosinophils were incubated, for 24 hours, with different concentrations of tacrolimus in the presence or absence of calcium ionophore, known to activate eosinophils. Supernatants were harvested and assessed for IL-8 by ELISA. Cell lysate was also processed for measurement of stored IL-8. Additionally, total RNA was measured in eosinophils by RT-PCR. The results showed tacrolimus inhibited IL-8 release, stored IL-8 in cells activated with calcium ionophore (Figures 1A and 1B), and IL-8 mRNA levels (Figure 1C). However, there was no significant effect on spontaneous release (unstimulated cells) of IL-8.

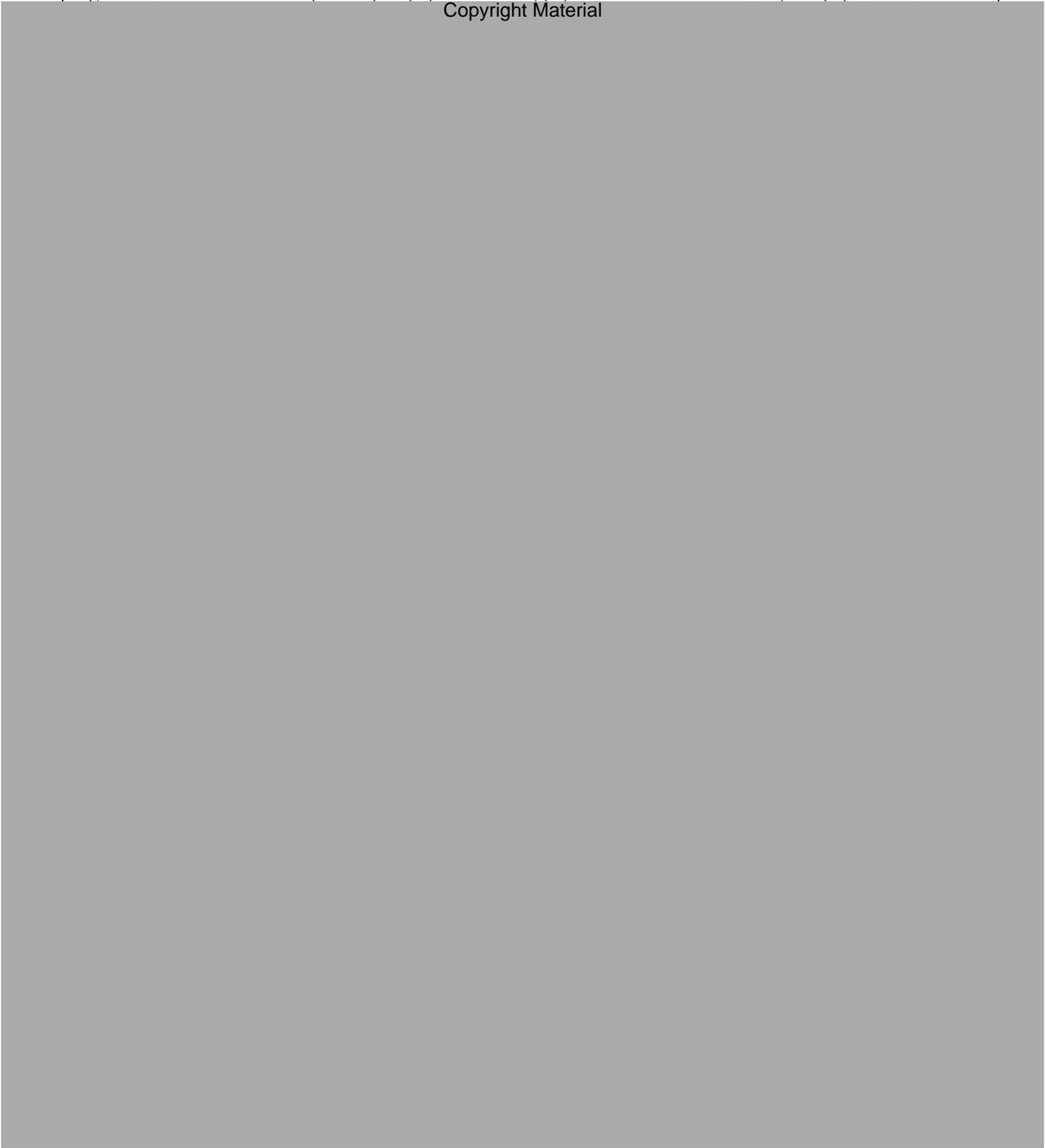
*Comments:*

*The study suggests that tacrolimus decreased IL-8 levels that may prevent infiltration of inflammatory cells by, at least in part, inhibiting chemokine release from eosinophils.*

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<sup>3</sup> Kohyama T, Takizawa H, Kawasaki S, Akiyama N, Sato M, Ito K, and Yamamoto K. A potent immunosuppressant FK506 inhibits IL-8 expression in human eosinophils. *Mol Cell Biol Res Comm* (1999) 1: 72- 77.

Figure 1: Effect of tacrolimus (FK506) on (A) IL-8 release, (B) intracellular levels of IL-8, and (C) IL-8 mRNA  
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- **Lemster *et al.*, 1995**

Lemster *et al.* (1995),<sup>4</sup> reported the effect of tacrolimus (0.1 – 0.3 mg/kg, daily) treatment on IL-8, and other cytokines as well as IL-8 receptor (IL-8R) in patients with severe, recalcitrant, chronic plaque psoriasis treated. Plasma specimens collected at different time points were measured for circulating IL-8 levels by ELISA. Pre-treatment IL-8 levels in plasma, ranged from < 11 to 3251 pg/ml (median 739 pg/ml); in two of the patients, IL-8 levels were below the sensitivity limit of the assay. After 3 months of tacrolimus therapy, there was a substantial reduction in the median IL-8 level; this reduction was sustained at 9 months (Table 3). However, the IL-8 levels were higher than those observed for normal individuals (range < 11 to 202 pg/mL) which could be due to increased inflammation in patients with psoriasis. At each time point, the variability in IL-8 levels was considerable.

Table 3: IL-8 plasma levels in relation to psoriasis area and severity index (PASI) score  
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Total cellular RNA was isolated from frozen skin biopsy specimens, collected before and after treatment with tacrolimus, and processed for measurement of cytokine (IFN- $\gamma$ , IL-6, IL-8, IL-10,

<sup>4</sup> Lemster BH, Carroll P B, Rilo HR, Johnson N, Nikaein A, and Thomson AW. IL-8/IL-8 receptor expression in psoriasis and the response to systemic tacrolimus (FK506) therapy. *Clin Exp Immunol* (1995) 99: 148-154.

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IL-1 $\beta$ , TNF- $\alpha$ , and TGF- $\beta$ ) and IL-8 receptor gene expression by RT-PCR. Treatment with tacrolimus reduced expression of IFN- $\gamma$ , IL-6, IL-10, and IL-1 $\beta$  (Figure 2). IL-8 gene expression was observed in specimens collected prior to treatment but not in any of the 4 patients post-treatment biopsies (Figure 2). TGF- $\beta$  was decreased in 2 patients (Patients 1 and 3), no effect in one patient (Patient 2) and increased in one patient (Patient 4). There appears to be no effect on TNF- $\alpha$  and IL-8 receptor expression after treatment (Figure 2). IL-2 and IL-4 were not detectable either pre-or post-transplant in any of the 4 patients (Figure 2).

*Comments:*

- *Treatment with tacrolimus reduced expression of IL-1 $\beta$ , IL-6, IL-8, IL-10, and IFN- $\gamma$ .*
- *There appears to be no effect on TNF- $\alpha$  and IL-8 receptor expression after treatment.*
- *IL-2 and IL-4 were not detectable either pre-or post-treatment. This could be due to the immune status of the patients with psoriasis as tacrolimus is known to inhibit production of IL-2 and IFN- $\gamma$  (see labeling).*

Figure 2: Cytokine gene expression determined by reverse transcriptase polymerase chain reaction (RT-PCR) in skin biopsies obtained pre- and post-treatment (60-150 days) from four psoriasis patients.

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- **Jiang *et al.*, 1999**

Jiang *et al.* (1999)<sup>5</sup> reported the effect of treatment with tacrolimus (3.2 mg/kg/day administered from days 5 to 10 post-transplant) on cytokine gene expression in a rat heterotopic heart transplant model. This regimen was shown to prolong heart allograft survival (a median survival time of 17 days). The local heart tissues and/or sera from the heart recipients at days 5, 7, and 10, were examined for the cellular and molecular events of immune response to heart allografts by immunohistochemistry, RT-PCR, and/or ELISA. In the allografts from untreated rats, various cytokine and cytotoxic factor mRNAs [IL-1 $\beta$ , IL-2, IL-4 (only marginally), IL-6, IL-10, IFN- $\gamma$ , TNF- $\alpha$ , TGF- $\beta$ , granzyme and perforin] were significantly up-regulated at day 5 compared to isograft controls. Both tacrolimus and CsA inhibited IL-1 $\beta$ , IL-2, IL-6, IFN- $\gamma$ , TNF- $\alpha$ , granzyme, and perforin mRNA expression in a range of from 20% to 50% at days 7 and 10 compared with the allograft control (data not shown); suppression of IL-10 expression was more in rats treated with tacrolimus than CsA (Figure 3). In serum, IL-10 was suppressed in rats treated with tacrolimus but not in those treated with CsA (Figure 3); however, both drugs suppressed IL-2 and IL-4 (data not shown).

*Comments:*

*Tacrolimus inhibited/decreased IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-10, IFN- $\gamma$ , TNF- $\alpha$ , TGF- $\beta$ , granzyme, and perforin mRNA expression levels. IL-10 levels were decreased in serum as well.*

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<sup>5</sup> Jiang H, Yang X-F, Soriano R, Fujitsu T, and Kobayashi M. Inhibition of IL-10 by FK 506 may be responsible for overcoming ongoing allograft rejection in the rat *Transplantation Proceedings* (1999) 31: 1203–1205.

Figure 3: Effect of tacrolimus on IL-10 mRNA in heart tissues and protein levels in serum of isograft and allograft

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- **Khanna *et al.*, 1999**

A study by Khanna *et al.* (1999)<sup>6</sup> reported the effect of tacrolimus (10 ng/mL) on IL-2 and TGF- $\beta$  production by purified T-cells, with and without activation with phorbol myristate acetate (PMA) (10 ng/ml) and ionomycin (400 ng/ml); tacrolimus (10 ng/mL) was added 30 minutes before activation. Cells were collected and processed for IL-2 and TGF- $\beta$  mRNA measurements by Northern blot; supernatants were collected for cytokine measurement by ELISA. Activation of T-cells resulted in IL-2 mRNA overexpression, and tacrolimus completely inhibited the induced expression (Figure 4A). The effect of tacrolimus on TGF- $\beta$  mRNA expression was opposite to that on IL-2 mRNA expression i.e., TGF- $\beta$  expression increased after incubation with tacrolimus (Figure 4A).

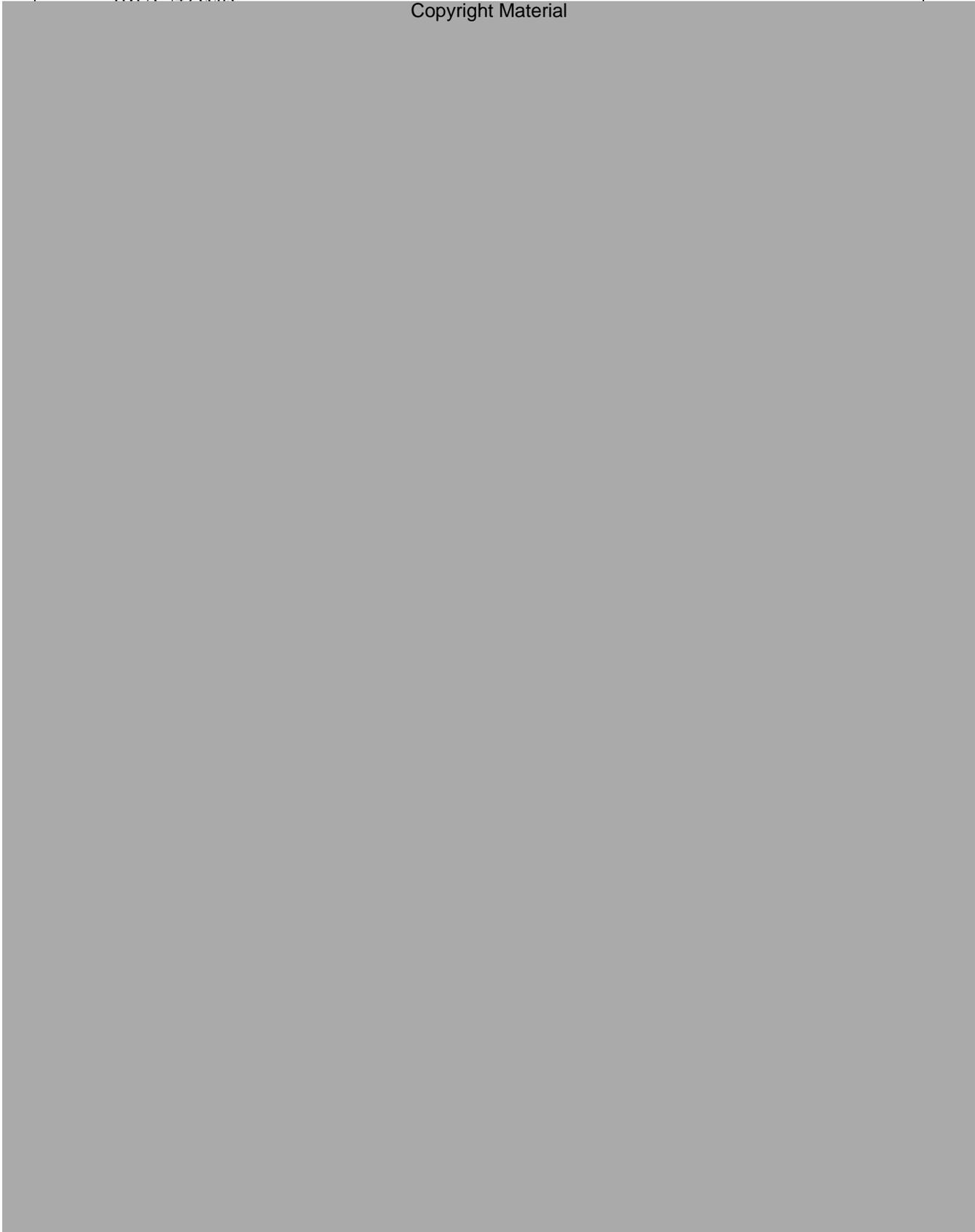
A-549 cells (a human lung adenocarcinoma cell line; nonlymphoid cells), incubated with tacrolimus (50 to 500 ng/mL) were also tested. Tacrolimus stimulated induction of TGF- $\beta$  expression by 3-5-fold in nonlymphoid cells (Figure 4B). Such an increase was concentration-dependent.

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<sup>6</sup> Khanna A, Cairns V, and Hosenpud JD. Tacrolimus induces increased expression of transforming growth factor-[beta]1 in mammalian lymphoid as well as nonlymphoid cells. *Transplantation* (1999) 67 (4): 614-619.

Figure 4: Effect of tacrolimus stimulates TGF- $\beta$  mRNA expression in (A) activated normal human T-cells and (B) A-549 cells

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TGF- $\beta$  protein secreted by purified activated human T-cells, peripheral blood mononuclear cells or A-549 cells incubated in the presence or absence of tacrolimus was quantified by sandwich ELISA. TGF- $\beta$  protein concentration in the culture supernatants of resting T-cells or activated T-cells was  $48 \pm 41$  and  $192 \pm 11$  ng/mL, respectively. In the presence of tacrolimus, TGF- $\beta$  concentration increased to  $560 \pm 63$  ng/mL (Figure 5).

Figure 5: Effect of tacrolimus on TGF- $\beta$  produced by human T-cells  
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TGF- $\beta$  protein was also increased by tacrolimus in human peripheral blood mononuclear cells regardless of the stimulant (anti-CD3, PHA, or ConA) used (Table 4). The effect of tacrolimus (8.6 ng/ml) on the anti-CD3-, concanavalin A-, and phytohemagglutinin- stimulated proliferation of peripheral blood mononuclear cells was also evaluated. The mean  $\pm$  SEM of the counts per minute (cpm) in the presence of tacrolimus were decreased compared to drug free cultures (Table 4) suggesting inhibitory effect on cell proliferation.

Table 4: Effect of tacrolimus on peripheral blood mononuclear cells proliferation and TGF- $\beta$  secretion  
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Incubation of A-549 cells with 50 ng/ml tacrolimus resulted in the inhibition of DNA synthesis; addition of anti-TGF- $\beta$  antibody (15  $\mu$ g/ml) reversed this inhibition, suggesting that TGF- $\beta$  induced by tacrolimus was biologically active (Figure 6).

Figure 6: Effect of tacrolimus on DNS synthesis in the presence or absence of anti-TGF- $\beta$  antibody (Ab)  
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*Comments:*

*Tacrolimus induced TGF- $\beta$  production. This might provide a complementary mechanism for its immunosuppressive properties.*

• **Sakuma *et al.*, 2001**

Sakuma *et al.* (2001)<sup>7</sup> reported the effect of tacrolimus on production of cytokines (IL-2, IL-3, IL-4, IL-5, IFN- $\gamma$ , and GM-CSF) by human PBMC and enriched T-cells from normal healthy volunteers. To stimulate T-cells directly without the effects of antigen-presenting cells, stimulating antibodies were fixed to microplate through anti-Fc antibody and stimulated with anti-CD3/CD2 antibody or anti-CD3/CD28 antibody. Cells were incubated with and without tacrolimus for 24 hours and supernatants collected for cytokine measurements by ELISA. Tacrolimus inhibited the production of IL-2, IL-3, IL-4, IL-5, IFN- $\gamma$ , and GM-CSF from both anti-CD3/CD2 and anti-CD3/CD28 stimulated cells (Table 5A). Tacrolimus IC<sub>50</sub> values were lower than that of steroids (Table 5B).

<sup>7</sup> Sakuma S, Higashi Y, Sato N, Sasakawa, T, Senogoku T, Ohkubo Y, Amaya T, and Goto, T. Tacrolimus suppressed the production of cytokines involved in atopic dermatitis by direct stimulation of human PBMC system. (Comparison with steroids). *Int Immunopharmacol* (2001) 1 (6): 1219-1226.

Table 5: IC<sub>50</sub> values (ng/mL) of tacrolimus in cytokine production from human PBMC  
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*Comments:*

*Tacrolimus inhibited the production of IL-2, IL-3, IL-4, IL-5, IFN- $\gamma$ , and GM-CSF. Overall, the study suggests an effect on both TH1 (IL-2 and IFN- $\gamma$ ) and Th2 (IL-4 and IL-5) response. Additionally, inhibition of IL-3 and GM-CSF suggest an effect on maturation and activation of mast cells and eosinophils. The activity of tacrolimus was higher than steroids.*

**2.2. Effect on IL-2 receptor expression on lymphocytes**

Kino *et al.* (1987)<sup>1</sup> reported the effect of tacrolimus on expression of IL-2 receptors. Briefly, different concentrations of tacrolimus, CsA or medium were added to the MLR cultures of normal human peripheral blood lymphocytes (PBL). After 6 days of culture the cells were harvested, washed and stained with fluorescein iso-thiocyanate (FITC)-conjugated CD25 (anti-Tac monoclonal antibody) and propidium iodide (for determining live cells). Cultivated live cells determined by the exclusion of propidium iodide were then analyzed on a FACS Analyzer. IL-2 receptor expression in the human MLR was determined by the percent of positive cells. The results showed that both tacrolimus and CsA inhibited the expression of IL-2 receptor on lymphocytes (Figure 7); the tacrolimus IC<sub>50</sub> values (0.1 nM i.e., 82.20 pg/mL) were about 10-fold lower than CsA.

Figure 7: Suppression of IL-2 receptor expression in human MLR.

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*Comments:*

*The study showed that tacrolimus inhibited expression of IL-2 receptors.*

### 2.3. Effect on nitric oxide and NF- $\kappa$ B

- *Conde et al., 1995*

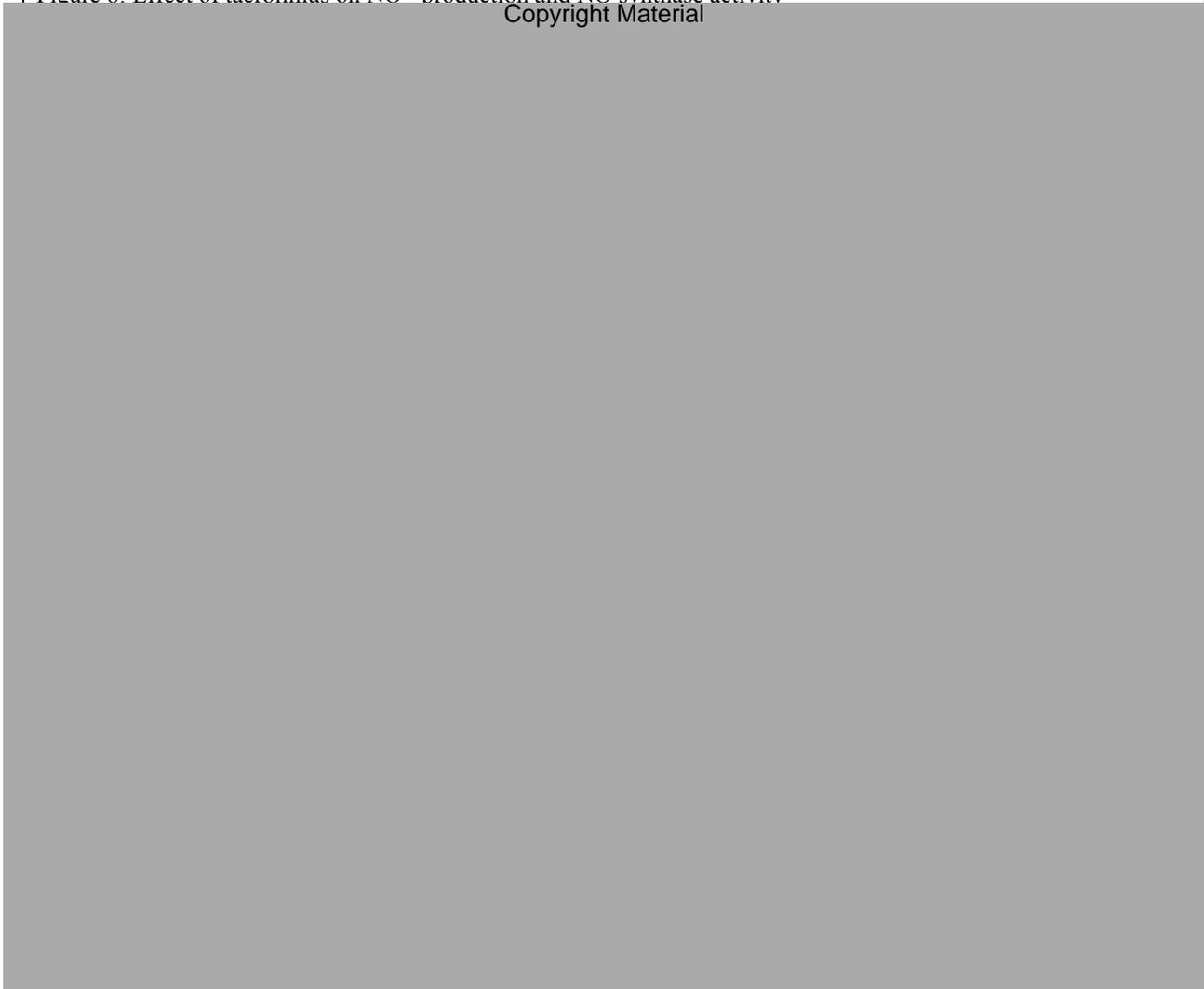
Conde *et al.* (1995)<sup>8</sup> reported the effect of tacrolimus on nitric oxide (NO) and NO synthase (NOS) production by macrophages. NO has been identified as a potent and pleiotropic mediator in several processes such as endothelium-dependent vasodilation, neurotransmission, and defence against parasites and other microorganisms. NO is synthesized by the oxidation of one of two chemically equivalent guanidinium nitrogens of L-arginine and the reaction is catalyzed by NOS. Nitrite (NO<sub>2</sub><sup>-</sup>) production (which is formed by the spontaneous oxidation of NO) by cultured macrophages was measured. Briefly, casein activated murine peritoneal macrophages were incubated with and without tacrolimus. The results showed tacrolimus and CsA inhibited NO<sub>2</sub><sup>-</sup> production after 48 hours of incubation (Figure 8A).

In another experiment, the effect of tacrolimus on nitric oxide synthase (NOS) activity was measured in the cytosolic fraction of activated macrophages. Maximal NOS activity was observed in activated macrophages. The results showed concentration dependent inhibitory effect of both tacrolimus and CsA on NOS activity (Figure 8B). The inhibitory effect of CsA was higher than that of tacrolimus on NOS activity. These data are consistent with those observed in cultured macrophages (Figure 8A).

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<sup>8</sup> Conde M, Andrade J, Bedoya FJ, Maria S, and Sobrino S. Inhibitory effects of cyclosporin A and FK-506 on nitric oxide production by cultured macrophages: evidence of a direct effect on nitric oxide synthase activity. *Immunology* (1995) 84: 476-481.

Figure 8: Effect of tacrolimus on NO<sup>-</sup> production and NO synthase activity  
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*Comments:*

*Tacrolimus inhibited NO generation and NOS activity.*

• **Kaibori *et al.*, 1999**

Kaibori *et al.*, (1999)<sup>9</sup> reported similar observations to those by Conde *et al.*, (1995)<sup>8</sup> using hepatocytes incubated with IL-1 $\beta$ . Briefly, rat hepatocytes were stimulated with IL-1 $\beta$  in the presence or absence of different concentrations of tacrolimus or CsA for up to 24 hours. Release of NO<sub>2</sub><sup>-</sup>, and level of NOS protein by Western blot, NOS mRNA and NF- $\kappa$ B subunits (p65 and p50) by Northern blot, as well as activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) by electrophoretic mobility shift assay (EMSA) on polyacrylamide gel were measured. The results showed that tacrolimus inhibited NO generation, NOS protein and mRNA expression after incubation with

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<sup>9</sup> Kaibori M, Sakitani K, Oda M, Kamiyama Y, Masu Y, Nishizawa M, Ito S, and Okumara T. Immunosuppressant FK506 inhibits inducible nitric oxide synthase gene expression at a step of NF-K-B activation in rat hepatocytes. *J Hepatol* (1999) 30: 1138-1145.

ENVARUSUS-XR

Veloxis Pharmaceuticals, Inc

IL-1 $\beta$  (Figure 9); such an effect was concentration and time-dependent. CsA was not effective. Tacrolimus was most effective at 10  $\mu$ M (8.22  $\mu$ g/mL) concentration showing 60% inhibition. The viability of cells was not affected by tacrolimus or CsA.

Figure 9: Effect of FK506 and CsA on induction of NO formation by IL-1 $\beta$  in hepatocytes  
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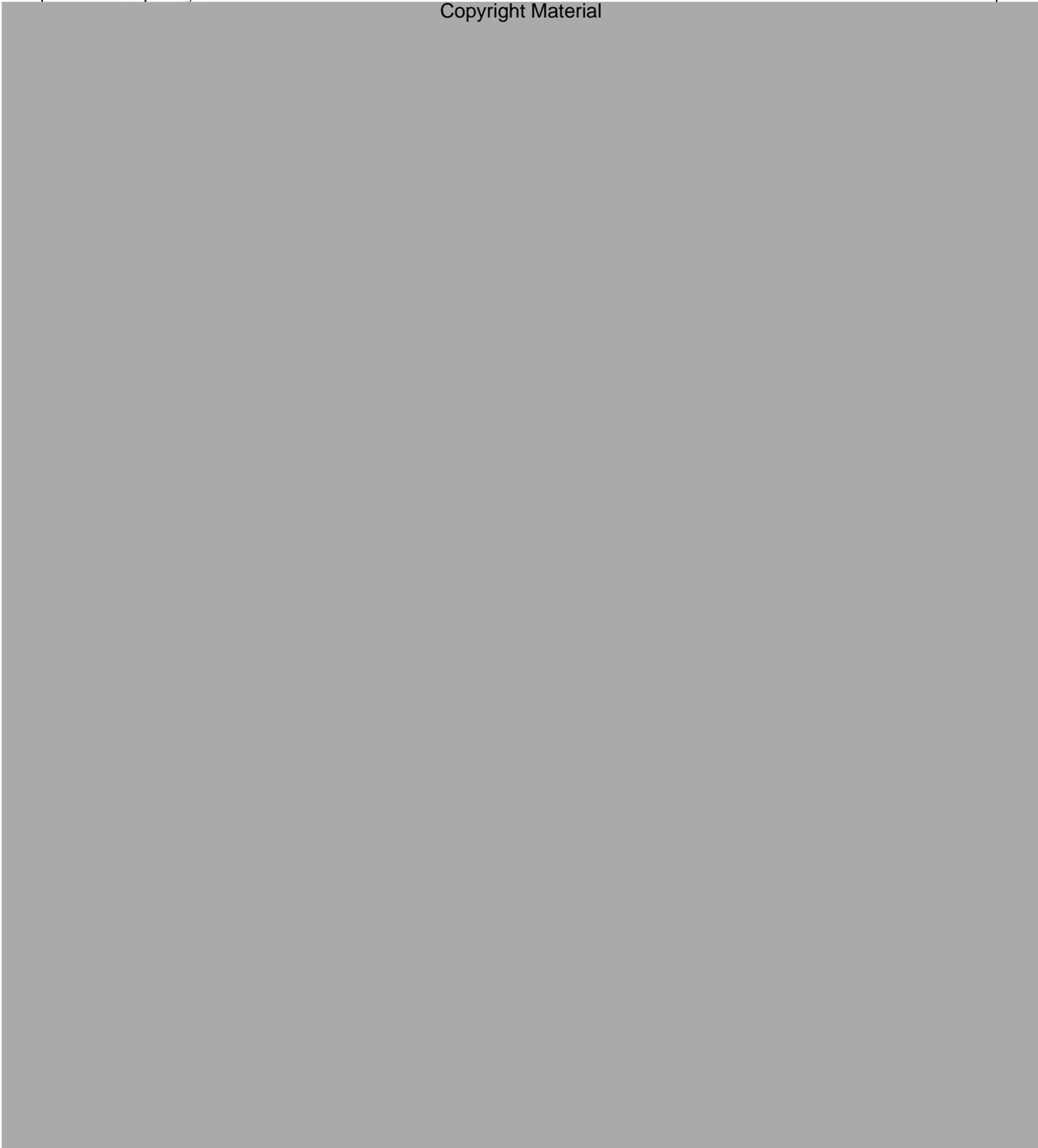
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Tacrolimus inhibited the NF- $\kappa$ B activation at 1 to 3 hours (Figure 10A) including mRNA levels of the p50/p65 subunits of NF- $\kappa$ B at 4 to 8 hours and 6 to 8 hours, respectively (Figure 10B). CsA had no inhibitory effect; it appears that CsA appears to increase mRNAs at 4 to 6 hours (Figure 10B).

Figure 10: Effect of FK506 and CsA on NF- $\kappa$ B activation and p65/p50 mRNA subunits induced by interleukin 1 $\beta$  in hepatocytes.

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*Comments:*

*The study suggests that tacrolimus modulates NO pathway by inhibiting induction of inducible NO synthase, and NK- $\kappa$ B activation, and the induction of p50 and p65 mRNAs.*

*CsA (10  $\mu$ M) had no inhibitory effect on the NO formation by rat hepatocytes induced by IL-1 $\beta$ . However, another study (Kunz et al., 1995<sup>10</sup>) reported an inhibitory effect of CsA on IL-1 $\beta$ -induced expression of iNOS using rat renal mesangial cells. It is unclear if such differences in activity of tacrolimus and CsA are due to different cell types or other experimental conditions.*

#### **2.4. Effect on apoptosis**

Migita et al. (1995)<sup>11</sup> reported an enhancement of apoptosis with tacrolimus (2 mg/kg) administered in combination with anti-CD3 antibody (30  $\mu$ g) or staphylococcal enterotoxin B (SEB; SEB is known to provoke a rapid expansion and subsequent apoptosis of SEB-reactive splenic V $\beta$ 8<sup>+</sup> T-cells) to mice. CsA (50 mg/kg) was used as a comparator. Thymocytes and/or V $\beta$ 8 and V $\beta$ 6 enriched cells were lysed and processed for evaluating DNA fragmentation by electrophoresis and phenotyping by flow cytometry. Additionally, splenocytes were processed for phenotyping by flow cytometry and for enrichment of CD3 positive T-cells by negative selection as well as DNA fragmentation by electrophoresis.

The results showed that administration of tacrolimus alone did not induce DNA fragmentation of thymocytes. However, tacrolimus increased DNA fragmentation in mice administered anti-CD3 antibody in combination with tacrolimus (Figure 11). CsA did not induce any DNA fragmentation. Similar observations were made in SEB injected mice; DNA fragmentation was observed in SEB-reactive V $\beta$ 8-positive thymocytes and not in SEB-reactive V $\beta$ 6-positive thymocytes (Figure 11).

---

<sup>10</sup> Kunz D, Walker G, Eberhardt W, Nitsch D, and Pfeilschifter J. Interleukin 1 $\beta$ -induced expression of nitric oxide synthase in rat renal mesangial cells is suppressed by cyclosporin A. *Biochem Biophys Res Commun* (1995) 216: 438-446.

<sup>11</sup> Migita K, Eguchi K, Kawabe Y, Tsukada T, Mizokami A, and Nagataki S. FK506 augments activation-induced programmed cell death of T lymphocytes in vivo. *J Clin Invest* (1995) 96: 727-732.

Figure 11: Effect of tacrolimus on DNA fragmentation in thymocytes.

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Anti-CD3 antibody treatment reduced CD4 and CD8 cells in the thymus. A combination of anti-CD3 + tacrolimus led to a marked deletion of CD4<sup>+</sup> CD8<sup>+</sup> immature thymocytes compared with that observed with anti-CD3 antibody alone (Figure 12). However, CsA treatment did not enhance the deletion of CD4<sup>+</sup> CD8<sup>+</sup> thymocytes induced by anti-CD3 antibody.

Figure 12: Effect of tacrolimus on thymocytes by flow cytometry

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The percentage of the apoptotic T-cells with hypodiploid DNA content was measured by flow cytometry. The authors state that that an increase in SEB-primed T-cells with hypodiploid DNA in FK506-treated mice was observed compared to control (data not shown).

In another experiment, mice were administered tacrolimus or CsA 3 days after administration of SEB and splenocytes prepared on day 7. The results showed a decrease in SEB-reactive V $\beta$ 8<sup>+</sup> T-cells, but not V $\beta$ 6<sup>+</sup> T-cells, in mice treated with tacrolimus (Table 6).

Table 6: Tacrolimus enhances elimination of CD4<sup>+</sup> V $\beta$ 8<sup>+</sup> T-cells in the spleen of SEB-treated mice

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Overall, the study suggests that tacrolimus increases apoptosis of antigen-stimulated thymocytes and peripheral T-cells.

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Another study by the same group (Migita *et al.*, 1999)<sup>12</sup> reported enhancement of apoptosis using murine splenic T-cells activated *in vivo* by administration of SEB. Tacrolimus (2 mg/kg) or CsA (50 mg/kg; used in some of the experiments) was administered intraperitoneally 36 hours after administration of SEB and spleens processed for preparation of single cell suspension and enrichment of V $\beta$ 8-positive T-cells. Cells were processed for phenotyping by flow cytometry and preparation of DNA to examine DNA fragmentation by electrophoresis. The results showed no effect of tacrolimus treatment on the expansion of CD4 V $\beta$ 8<sup>+</sup> T-cells; CD8 V $\beta$ 8<sup>+</sup> T-cells were slightly reduced (Figure 13A). Fas expression in both CD4 and CD8 subsets (Figure 13B) was also not altered thereby suggesting that Fas expression was not involved in inducing tacrolimus mediated apoptosis.

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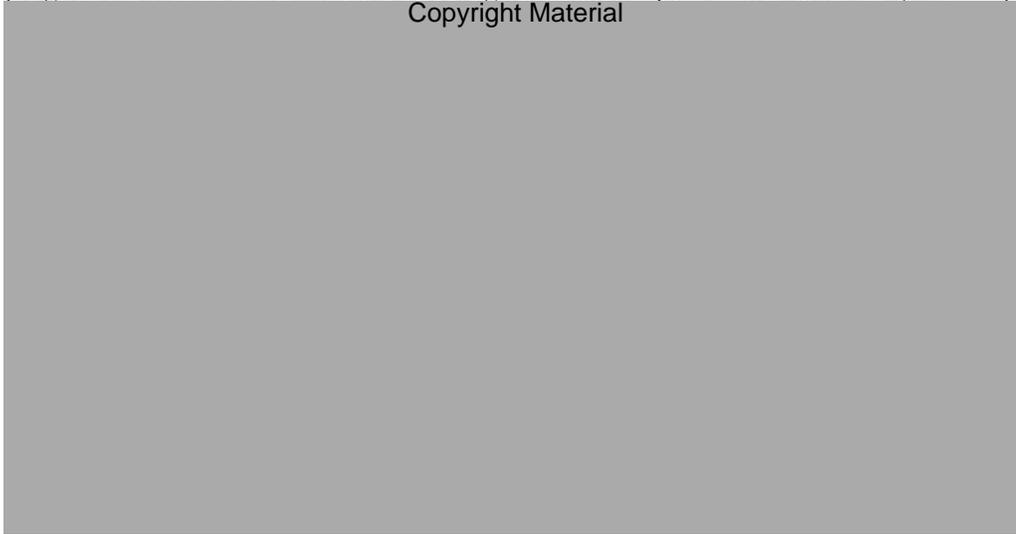
<sup>12</sup>Migita K, Origuchi T, Kawabe Y, Tominaga M, Hiroaki I, Kawakami A, and Eguchi K. FK506 markedly enhances apoptosis of antigen-stimulated peripheral T cells by down-regulation of Bcl-x<sub>L</sub>. *Transplantation* (1999) 68 (7): 1018-1023

Figure 13: Effect of tacrolimus on spleen cells by flow cytometry  
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Similarly to that reported in the study (Migita *et al.*, 1995<sup>11</sup>) summarized above, tacrolimus treatment increased DNA fragmentation in SEB-activated V $\beta$ 8<sup>+</sup> T-cells (Figure 14); however, no DNA fragmentation was observed in cells from mice treated with CsA-treated and was similar to that of SEB-activated V $\beta$ 8<sup>+</sup> T-cells.

Figure 14: Effect of tacrolimus on DNA fragmentation in  $V\beta 8^+$  T-cells from the thymus  
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The apoptotic signal is regulated by a number of intracellular factors that either promote or prevent apoptosis. The expression of anti-apoptotic mediators (Bcl-2 and Bcl-x<sub>L</sub>) and pro-apoptotic mediator (Bax) was analyzed by western blot. Tacrolimus administration did not alter the level of Bcl-2 in  $V\beta 8^+$  T-cells (Figure 15A). Bcl-x<sub>L</sub> expression returned to basal level at 12 hours after treatment with tacrolimus (Figure 15B). In another experiment, mice were killed 4, 8, and 12 hours after tacrolimus treatment, and splenic  $V\beta 8^+$  T-cells isolated. Down-regulation of Bcl-x<sub>L</sub> expression on SEB-activated  $V\beta 8^+$  T-cells was observed at 8 hours that increased at 12 hours (Figure 15C). There was no effect on Bax expression (Figure 15D), known to antagonize the function of Bcl-2 and Bcl-x<sub>L</sub>. CsA was not tested for measurement of Bcl, Bcl-x<sub>L</sub>, or Bax.

Figure 15: Effect of tacrolimus treatment on expression of Bcl-2, Bcl-xL, and Bax expression on splenic V[beta]8+ T-cells

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*Comments:*

*The study suggests that tacrolimus induces SEB-activated T-cell apoptosis in vivo by down-regulating an anti-apoptotic protein, Bcl-x<sub>L</sub>. Fas expression is not altered.*

*Apoptosis is important for maintaining cellular homeostasis and may represent a potential mechanism for immunosuppression.*

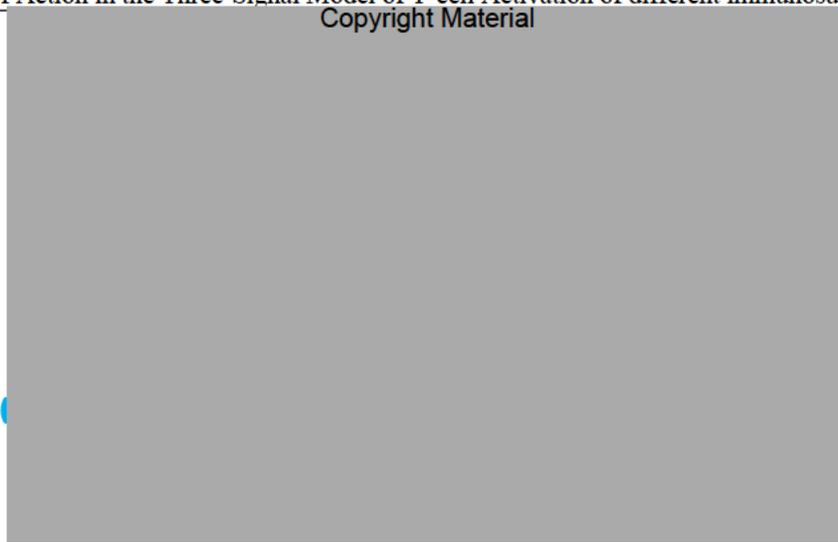
### **3. Conclusions**

Studies showed that tacrolimus inhibited T-cell activation, generation of cytotoxic lymphocytes as well as T-cell dependent B-cell proliferation and thereby down-regulates the processes leading to acute graft rejection. Tacrolimus inhibited not only IL-2 and IFN- $\gamma$  but also the expression and/or production of several other cytokines that include IL-1 $\beta$ , IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, TNF- $\alpha$ , and GM-CSF. IL-2 receptor expression and NO release were also inhibited. However, apoptosis and TGF- $\beta$  expression were increased that may further enhance the immunosuppressive activity of tacrolimus.

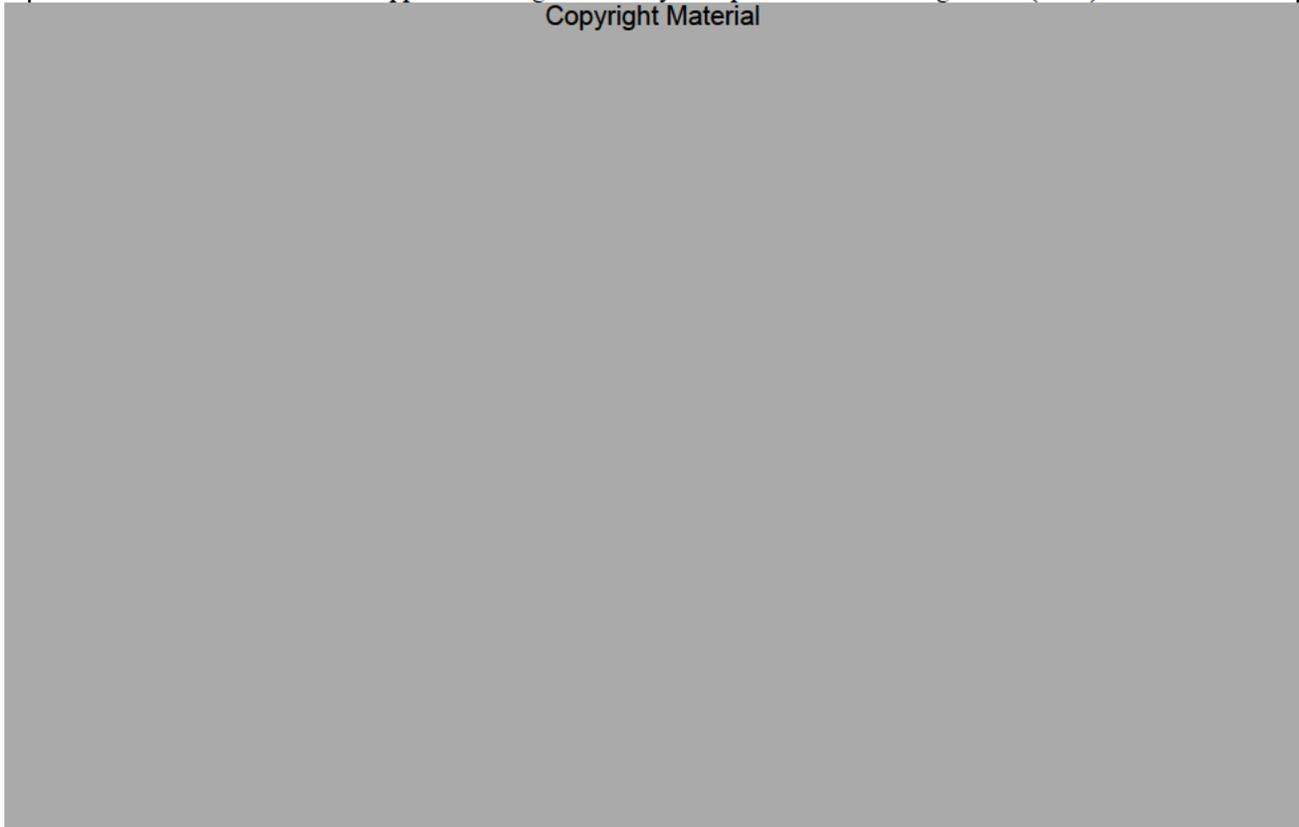
Tacrolimus may exhibit its immunosuppressive activity by a variety of different mechanisms (Figure 16). The primary event appears to be formation of a complex between tacrolimus and the FK-506 binding protein (FKBP) 12 that binds to calcineurin, inhibiting its phosphatase activity. Inhibition of calcineurin activity leads to prevention of dephosphorylation and translocation of various nuclear factors such as the cytosolic subunit of NF-AT and NF- $\kappa$ B to the nucleus (Figure 16). NF- $\kappa$ B, a group of dimeric transcription factors, exerts both positive and negative effects on gene transcription and has a large impact on the development, homeostasis, survival, and function of T-cells.

Calcineurin is widely distributed in mammalian tissues and it is possible that inhibition of calcineurin may be responsible for some of the well-established effects including nephrotoxic, diabetogenic, neurological and cardiovascular effects (Table 7) where several cellular and immunological mechanisms are potentially involved.

Figure 16: Sites of Action in the Three-Signal Model of T-cell Activation of different immunosuppressive drugs  
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Source: Halloran PF. Immunosuppressive drugs for kidney transplantation. *New Eng J Med* (2004) 351: 2715-2729.  
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Source: <https://www.pharmgkb.org/pathway/PA165985892>  
Citation: Barbarino Julia M, Staatz Christine E,  
Venkataramanan Raman, Klein Teri E, Altman Russ B.  
"PharmGKB summary: cyclosporine and tacrolimus  
pathways" *Pharmacogenetics and genomics* (2013).

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Table 7: Overview of the actions of tacrolimus that may contribute to its pharmacodynamic effects

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TAC = tacrolimus

Source: Scott *et al.* (2003) Tacrolimus: A further update of its use in the management of organ transplantation. *Drugs* 63 (12) 1247-1297.

## 4. The Labeling

### 4.1. The applicant's version of the labeling

The applicants proposed labeling is (b) (4) that reads as follows:

#### 12.1 Mechanism of Action

(b) (4)

#### Comments:

- *The labeling proposed by the applicant is (b) (4). Since then several published studies showed inhibitory activity of tacrolimus against not only IL-2 and IFN- $\gamma$  but also IL-1 $\beta$ , IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, TNF- $\alpha$ , and GM-CSF. IL-2 receptor expression and NO release are also inhibited. However, apoptosis and TGF- $\beta$  expression are increased. Such information should be added to the labeling.*
- *Calcineurin is widely distributed in mammalian tissues. A complex of tacrolimus-FKBP-12, calcium, calmodulin, and calcineurin is then formed and the phosphatase activity of calcineurin prevents not only the dephosphorylation and translocation of various nuclear factors such as the cytosolic subunit of nuclear factor of activated T-cells (NF-AT) but also NF- $\kappa$ B to the nucleus. Inhibition of NF- $\kappa$ B should be added to the labeling.*

### 4.2. FDA's version of the labeling

(Additions are double-underlined and deletions striked out)

Tacrolimus (b) (4) binds to an intracellular protein, FKBP-12. A complex of tacrolimus-FKBP-12, calcium, calmodulin, and calcineurin (an ubiquitous mammalian intracellular enzyme) is then formed and the phosphatase activity of calcineurin inhibited. Such inhibition (b) (4) prevents the dephosphorylation and translocation of various factors such as the nuclear factor of activated T-cells (NF-AT) and nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- $\kappa$ B).

Tacrolimus inhibits the expression and/or production of several cytokines that include (b) (4) interleukin (IL)-1 beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, gamma interferon, tumor necrosis factor-alpha, and granulocyte macrophage colony stimulating factor). Tacrolimus also inhibits IL-2 receptor expression and nitric oxide release, induces apoptosis and production of transforming growth factor-beta that can lead to immunosuppressive activity. The net result is the-inhibition of T-lymphocyte activation and proliferation as well as T-helper-cell-dependent B-cell response (i.e., immunosuppression).

## 5. Recommendations

This NDA is approvable with respect to Immunology/Microbiology pending an accepted version of the labeling. For changes to the labeling see section 4.2 above.

*Shukal Bala*  
Shukal Bala, Ph.D.  
Microbiologist/Immunologist

**CONCURRENCE:**  
Division Director/Renata Albrecht, MD

CC:  
DTOP/NDA 206406  
DTOP/PM/Lois Almoza

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/s/  
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SHUKAL BALA  
07/23/2014

RENATA ALBRECHT  
07/24/2014

## IMMUNOLOGY/MICROBIOLOGY FILING CHECKLIST

**NDA Number:** 206406  
(SDN-1)

**Applicant:** Veloxi Pharmaceuticals  
Inc

**Stamp Date:** 12/28/ 2013

**Drug Name:** Tacrolimus extended release

**NDA Type:** NME

On **initial** overview of the NDA application for filing:

	<b>Content Parameter</b>	<b>Yes</b>	<b>No</b>	<b>Comments</b>
1	Is the immunology/microbiology information (preclinical/nonclinical and clinical) described in different sections of the NDA organized in a manner to allow substantive review to begin?	X		
2	Is the immunology/microbiology information (preclinical/nonclinical and clinical) indexed, paginated and/or linked in a manner to allow substantive review to begin?	X		
3	Is the immunology/microbiology information (preclinical/nonclinical and clinical) legible so that substantive review can begin?	X		
4	On its face, has the applicant <u>submitted</u> <i>in vitro</i> data in necessary quantity, using necessary clinical and non-clinical strains/isolates, and using necessary numbers of approved current divisional standard of approvability of the submitted draft labeling?			N/A
5	Has the applicant <u>submitted</u> any required animal model studies necessary for approvability of the product based on the submitted draft labeling?			N/A
6	Has the applicant <u>submitted</u> all special/critical studies/data requested by the Division during pre-submission discussions?			N/A
7	Has the applicant <u>submitted</u> the clinical microbiology datasets in a format which intends to correlate baseline pathogen with clinical and microbiologic outcome?			N/A
8	Has the applicant <u>submitted</u> draft/proposed interpretive criteria/breakpoint along with quality control (QC) parameters and interpretive criteria, if applicable, in a manner consistent with contemporary standards, which attempt to correlate criteria with clinical results of NDA/BLA studies, and in a manner to allow substantive review to begin?			N/A
9	Has the applicant <u>submitted</u> a clinical microbiology dataset in an appropriate/standardized format which intends to determine resistance development by correlating changes in the phenotype (such as <i>in vitro</i> susceptibility) and/or genotype (such as mutations) of the baseline pathogen with clinical and microbiologic outcome?			N/A

**IMMUNOLOGY/MICROBIOLOGY FILING CHECKLIST  
NDA 206406**

	<b>Content Parameter</b>	<b>Yes</b>	<b>No</b>	<b>Comments</b>
10	Has the applicant used standardized or nonstandardized methods for measuring microbiologic outcome? If nonstandardized methods were used, has the applicant included complete details of the method, the name of the laboratory where actual testing was done and performance characteristics of the assay in the laboratory where the actual testing was done?			N/A
11	Has the applicant <u>submitted</u> draft labeling consistent with current regulation, divisional and Center policy, and the design of the development package?	X		
12	Has the applicant <u>submitted</u> annotated immunology/microbiology draft labeling consistent with current divisional policy, and the design of the development package?	X		
13	Have all the study reports, published articles, and other references been included and cross-referenced in the annotated draft labeling or summary section of the submission?	X		
14	Are any study reports or published articles in a foreign language? If yes, has the translated version been included in the submission for review?	X		

**IS THE MICROBIOLOGY SECTION OF THE APPLICATION FILEABLE? Yes**

If the NDA is not fileable from the microbiology perspective, state the reasons and provide comments to be sent to the Applicant. N/A

Please identify and list any potential review issues to be forwarded to the Applicant for the 74-day letter.  
None

Shukal Bala 2/14/14  
 Reviewing Microbiologist Date

Renata Albrecht 2/14/14  
 Division Director Date

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/s/  
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SHUKAL BALA  
02/14/2014

RENATA ALBRECHT  
02/14/2014

# MEMORANDUM



DEPARTMENT OF HEALTH AND HUMAN SERVICES  
PUBLIC HEALTH SERVICE  
FOOD AND DRUG ADMINISTRATION  
CENTER FOR DRUG EVALUATION AND RESEARCH

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**DATE:** 10 January 2014

**TO:** NDA 206406

**FROM:** Bryan S. Riley, Ph.D.  
Team Leader (Acting)  
OPS/New Drug Microbiology Staff

**THROUGH:** Stephen E. Langille, Ph.D.  
Master Review Microbiologist  
OPS/New Drug Microbiology Staff

**cc:** Lois A. Almoza  
Regulatory Project Manager  
OND/DTOP

**SUBJECT:** Product Quality Microbiology assessment of Microbial Limits for Envarsus (tacrolimus) [Submission Date: 30 December 2013]

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**The Microbial Limits specification for Envarsus is acceptable from a Product Quality Microbiology perspective. Therefore, this submission is recommended for approval from the standpoint of product quality microbiology.**

Envarsus is a Tablet for oral administration.

The drug product is tested for Microbial Limits at release using a method consistent with USP Chapter <61> (Microbiological Examination of Non-sterile Products: Microbial Enumeration Tests) and <62> (Microbiological Examination of Non-sterile Products: Tests for Specified Microorganisms). The Microbial Limits acceptance criteria are consistent with USP Chapter <1111> (Microbiological Examination of Non-sterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use).

# MEMORANDUM

**Table 1 – Microbial Limits Specifications**

<b>Test</b>	<b>Acceptance Criteria</b>
Total Aerobic Microbial Count (USP <61>)	NMT $10^3$ CFU/g
Total Yeast and Mold Count (USP <61>)	NMT $10^2$ CFU/g
<i>Staphylococcus aureus</i> (USP <62>)	Absent in 1 g
<i>Escherichia coli</i> (USP <62>)	Absent in 1 g
<i>Pseudomonas aeruginosa</i> (USP <62>)	Absent in 1 g
<i>Salmonella</i> species (USP <62>)	Absent in (b)(4)

The Microbial Limits test methods were verified to be appropriate for use with the drug product following procedures consistent with those in USP Chapter <61> and <62>.

The drug product will also be tested for Microbial Limits at 24 and 36 months as part of the post-approval stability protocol.

## ADEQUATE

**Reviewer Comments – The microbiological quality of the drug product is controlled via a suitable testing protocol.**

END

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/s/  
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BRYAN S RILEY  
01/13/2014

STEPHEN E LANGILLE  
01/13/2014