

Differential Inhibition of Primary versus Preactivated T Cells by Pimecrolimus but Not by Tacrolimus in vitro

Frank S. Kalthoff Anthony Winiski Petra Fichtinger Brigitte Schwendinger
Shirley Wang Cordula Weishaeupl Anton Stuetz

Novartis Institutes for Biomedical Research, Vienna, Austria

Key Words

Pimecrolimus • Tacrolimus • Primary response •
Recall response • T cell

Abstract

Background: Recent studies in murine models of allergic contact dermatitis have shown that systemic treatment with pimecrolimus in contrast to tacrolimus did not inhibit the sensitization phase, whereas both compounds equivalently suppressed the inflammatory response in sensitized animals. This finding indicated a differential sensitivity of antigen-naïve and primed T cells towards pimecrolimus and tacrolimus. **Methods:** T cells obtained from healthy and allergic donors were subjected to primary and secondary stimulation by allogeneic or staphylococcal superantigen-presenting dendritic cells (DC). Human skin-derived, allergen-specific T cell clones from an atopic dermatitis patient were activated by anti-CD3 antibodies or by specific allergen-presenting DC. The inhibition of T cell proliferation and cytokine release by graded doses of calcineurin inhibitors was evaluated. **Results:** Primary stimulation of T cells was inhibited by pimecrolimus with an approximately 8-fold lower potency as compared with tacrolimus. In contrast, the secondary response of ex vivo expanded T cells activated by allogeneic or staphylococcal superantigen-presenting DC was inhibited by both compounds with equivalent potency.

Likewise, both drugs showed very similar potency to inhibit the proliferation and cytokine synthesis from antigen-stimulated T cell clones and the induction of cytokines in Jurkat T cells. **Conclusion:** These data indicate that pimecrolimus has a selectivity for antigen-primed memory T cells not seen with tacrolimus.

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Introduction

Pimecrolimus and tacrolimus have been approved for the topical treatment of atopic dermatitis. Both drugs share the same mechanism of action. They bind with high affinity to macrophilin-12, the common cytosolic receptor. The complex between macrophilin-12 and either tacrolimus or pimecrolimus binds to calcineurin, a serine/threonine phosphatase, and inhibits its enzymatic activity [1, 2]. Calcineurin is also the target of the complex between cyclosporin A (CsA) and its intracellular receptor cyclophilin [3]. The inhibition of calcineurin prevents the nuclear translocation of the transcription factor NFAT, the nuclear factor of activated T cells [4], which is paramount to the activation of many genes specifically expressed after T cell stimulation, such as cytokines, cytokine receptors and adhesion molecules [5]. Previous in vitro studies reported that CsA was about 50- to 100-fold

less potent than tacrolimus and about 10-fold less potent than pimecrolimus [6–10]. However, data from studies comparing the two compounds side by side are lacking and there is no information on their effect in different T cell populations, such as unstimulated or naïve T cells and repeatedly activated memory T cells, the frequency of which can vary considerably in chronic immunological diseases. Interestingly, pimecrolimus and tacrolimus were shown to have a different pharmacologic profile in a mouse model of allergic contact dermatitis, a delayed-type cutaneous hypersensitivity (CHS) reaction. Whereas pimecrolimus and tacrolimus were equipotent in inhibiting the inflammatory response when administered orally at challenge, pimecrolimus in contrast to tacrolimus did not inhibit the sensitization towards the contact allergen, even at a 4-fold higher dose [11]. The present study shows that pimecrolimus has a more selective activity profile against primed T cells in vitro as compared with tacrolimus. It was observed to be significantly less potent than tacrolimus in inhibiting T cell proliferation and cytokine synthesis after primary T cell activation but equipotent against a secondary activation after expansion of the same T cell population. In line with these findings, pimecrolimus and tacrolimus showed a very similar potency to inhibit the stimulation of human T cell clones (TCC) derived from the skin of an atopic dermatitis patient.

Materials and Methods

Culture Medium, Reagents and Chemicals

The standard culture medium (SCM) was RPMI 1640 (Invitrogen Life Technologies, Grand Island, N.Y., USA), further supplemented with NaHCO_3 (2 mg/ml), penicillin and streptomycin (50 $\mu\text{g}/\text{ml}$ each), L-glutamine (2 mM) and 10% fetal calf serum (FCS; Invitrogen Life Technologies). The culture medium for the TCC restimulation assays and propagation differed from the SCM in that it contained 5% FCS and 5% human serum (HS; pool of sera from donors with blood group AB, purchased from PAA Laboratories, Linz, Austria), referred to as SCM-HS. The recombinant cytokines (Novartis Pharma AG, Basel, Switzerland) granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 had a specific activity of 6×10^6 U/mg, and IL-2 had a specific activity of 1×10^7 U/mg. Pimecrolimus, tacrolimus and CsA (Novartis Pharma AG) were dissolved in dimethylsulfoxide (pimecrolimus, tacrolimus) or ethanol (CsA) to yield 5-mM stock solutions. Phorbol 12-myristate 13-acetate (PMA) and phytohemagglutinin (PHA; Sigma-Aldrich, Austria) were dissolved at 10 mg/ml in DMSO (PMA) or 1 mg/ml SCM (PHA) and stored frozen. Anti-CD3 monoclonal antibody (mAb; HIT3a) and the anti-CD28 mAb (CD28.2) were purchased from Pharmingen, San Jose, Calif., USA, and the goat anti-mouse immunoglobulin G (IgG; Cappel) was obtained from MP Biomedicals, Inc., Aurora, Ohio, USA.

Cytokine Induction in Peripheral Blood Mononuclear Cells, Purified T Cells and Jurkat T Cells

Peripheral blood mononuclear cells (PBMC) were isolated from human leukapheresis samples by centrifugation over Ficoll-Hypaque (Amersham Biosciences) or Lymphoprep® (Axis Shield, Oslo, Norway) and stored frozen in 10% FCS and 10% DMSO. Aliquots of PBMC from 2 different donors were suspended in SCM and seeded into 96-well plates at a density of either 2.5×10^4 or 1×10^5 cells/well. The solvent control and the compounds were added 2 h prior to the stimulation by 100 ng/ml staphylococcal enterotoxin type B (SEB; Sigma), and the cells were incubated for 68 h prior to centrifugation and collection of the cell-free supernatant for subsequent multiplex analysis of secreted cytokines. T cells were purified from PBMC of another 3 donors using the pan T cell isolation kit (Miltenyi Biotech, Bergisch-Gladbach, Germany) and seeded into 96-well plates at 1×10^5 cells/well. Following preincubation by serial dilutions of calcineurin inhibitor (CNI) compounds for 1 h, T cells were stimulated by anti-CD3 and anti-CD28 mAb immobilized via plastic surface-coated goat anti-mouse IgG-specific antibodies. Cell-free supernatant was taken after 24 h for subsequent multiplex determination of cytokine levels (see below). Jurkat T cells were stimulated after preincubation with serial dilutions of CNI compounds in 24-well plates at a cell density of 1×10^6 cells/well using PMA and PHA at a final concentration of 50 ng/ml and 2 $\mu\text{g}/\text{ml}$, respectively. At the indicated time points (table 3), cell-free culture supernatants were harvested for subsequent cytokine determination by ELISA, and the cell pellets snap-frozen in liquid nitrogen and stored at -20°C until isolation of total RNA.

Antigen-Specific Human TCC

The CD4+ human TCC, referred to as CFTS4:3.1, CFTS4:2.15 and CFTS4:2.20, were established from punch biopsies of clinically noninvolved skin from an atopic dermatitis patient, taken at 24 h after epicutaneous challenge with a protein extract of house dust mites, as previously described [12]. The TCC recognize peptides of the major allergen Der p 1 of *Dermatophagoides pteronyssinus* (Dpt) in association with the major histocompatibility complex class II restriction molecule HLA-DPw4 (allele HLA-DPB1*0401), as previously reported [13]. The T cells were activated every 14 days with anti-CD3 mAb immobilized by plastic surface-bound goat anti-mouse IgG and expanded in SCM-HS supplemented with IL-2 and IL-4 (50 U/ml).

Generation of Dendritic Cells from Monocytes in vitro

Monocytes were purified from PBMC obtained from healthy donors by immunomagnetic separation techniques using the monocyte isolation kit (Miltenyi Biotech). Monocytes were enriched to a purity of 95% as determined by flow cytometry. Dendritic cell (DC) differentiation was induced by GM-CSF (600 U/ml) and IL-4 (300 U/ml), as described previously [14]. On day 6 of culture, DC were harvested and cell aliquots frozen in liquid nitrogen for later use. Batches of DC matching to the TCC with regard to HLA-DPw4 expression were preselected by testing DC of different donors for specific stimulation of house dust mite antigen-mediated proliferation of the TCC.

Allogeneic and Autologous Mixed Lymphocyte Response

Polyclonal T cells were obtained from PBMC of healthy donors applying negative immunomagnetic separation techniques. The

pan T cell isolation kit was used for preparation of all CD4+ plus CD8+ T cells, and purified CD4+ T cells were isolated with the CD4+ T cell isolation kit (Miltenyi Biotech). Pan T cells or purified CD4+ T cells were stimulated by autologous DC [auto-mixed lymphocyte response (MLR)] in the presence of a final concentration of 50 pg/ml of SEB or by allogeneic DC (allo-MLR). In each case, the MLR was conducted at a DC/T cell ratio of 1/40 in the absence of drug (control) and in the presence of pimecrolimus (300–0.045 nM), tacrolimus (30–0.0045 nM) or CsA (900–0.14 nM). T cell proliferation was measured by adding 1 μ Ci/well of tritiated thymidine (specific activity 3,000 mCi/mmol; Amersham, UK) during the last 16 h of a 4-day (auto-MLR) or 5-day (allo-MLR) culture period. The results are expressed as the mean counts per minute $\pm$ standard deviation of quadruplicate wells. T cells and monocyte-derived DC were seeded separately in order to control for background proliferation.

Primary versus Secondary MLR

T cells and DC were prepared from PBMC obtained from 4 healthy and 4 allergic donors. The 4 allergic donors (diagnosed at the General Hospital of the Medical University of Vienna, Vienna, Austria) gave consent to donate blood prior to receiving any medication. Two donors suffered from acute exacerbations of rhinoconjunctivitis, and another 2 presented with acute flares of atopic dermatitis. T cells were subjected to a primary stimulation in the allo- or auto-MLR. Parallel cocultures of DC and T cells were allowed to expand for a total of 11 days, and the proliferation was assessed after restimulation with either allogeneic or autologous SEB-treated fresh DC after 3 days (secondary MLR).

Induction of Proliferation and Cytokine Synthesis by TCC

Specific stimulation of TCC was mediated by DC incubated overnight in SCM supplemented with 50 μ g/ml of a protein extract of *Dpt*, containing 18.5 μ g of the major allergen Der p 1 per milligram *Dpt* protein (Artu Biologicals NV, Lelystad, The Netherlands). DC not incubated with antigen served as negative controls in the T cell stimulation assay. Following washing to remove excess antigen, DC and TCC were mixed and seeded into 96-well culture plates at a DC/T cell ratio of 1/40 in SCM-HS as culture medium. The compound concentration ranged between 160 and 0.6 nM for CsA, and between 20 and 0.078 nM for pimecrolimus and tacrolimus using 2-fold dilution steps. Each sample was tested in quadruplicate and the proliferation was determined as described for MLR experiments. To induce cytokine synthesis, the TCC were either stimulated using *Dpt*-pulsed monocyte-derived DC or, alternatively, 200 ng/ml of anti-CD3 mAb bound to preimmobilized goat anti-mouse IgG in the presence of 2 nM PMA. The compounds were tested over the concentration range of 30–0.0045 nM (pimecrolimus) or 10–0.0015 nM (tacrolimus), using 3-fold dilution steps. Cell-free supernatants were harvested after 48 h and analyzed by the Luminex-based multiplex analysis.

Cytokine Analyses

ELISA

In the Jurkat experiments, cytokine protein levels were measured using either FlexiaTM [tumor necrosis factor (TNF)- α , IL-2 and IL-8] or CytoSetsTM (GM-CSF and IL-3) ELISA kits (Bio-source Europe SA, Belgium) according to the manufacturer's recommendations.

Multiplex Analysis of Cytokine Production

The Luminex LabMAPTM (Laboratory Multi-Analyte Profiling) technology was used for all other cytokine determinations. The levels of IL-2, IL-3, IL-4, IL-5, IL-8, IL-10, TNF- α , interferon (IFN)- γ and GM-CSF were determined using the Fluorokine MAP multiplex detection system (Fluorokine MAP[®] base kit, R&D Systems, Minneapolis, Minn., USA) and the Luminex 100TM analyzer (Luminex, Austin, Tex., USA). The data were transferred to preformulated ExcelTM spreadsheets. Cytokine levels in samples were calculated from interpolation of the standard curve (point-to-point fit). The averages and standard deviations of the cytokine levels at each compound concentration were calculated, and dose-response curves were fitted using the Origin[®] (7 SR1) software (OriginLab Corporation, Northampton, Mass., USA) to the 4-parameter logistic equation.

Gene Expression Analysis by Real-Time Polymerase Chain Reaction

Total RNA was isolated from 1×10^6 Jurkat cells, using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. First-strand cDNA was synthesized from 250 ng of RNA, using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, Wisc., USA) and random hexamer primer (Promega); about 1% of the products of this reaction were used as a template for quantitative real-time polymerase chain reaction (PCR). Real-time PCR was performed on an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, Calif., USA) using TaqMan Universal Master Mix (Applied Biosystems). Primers and probe sequences were designed based on the sequences reported in GenBank with the Primer Express software (Applied Biosystems). The following sequences were used as forward primer (F), reverse primer (R) and labeled probe (P). IL-2: (F) TCACCAGGATGCTCACATTTAAGT, (R) TCCAGAGGTTTGAGTTCTTCTTCTAGA, (P) CCAAGAAGGCCACAGAACTGAAACAGCTT; IL-3: (F) TCGCGATCTTTTAGTCCAACGT, (R) TGACCCAGAGGTTTCAGAAAGTTC, (P) CAGCTCGTTC-TCTGGGCCTTCTCACC; IL-8: (F) CTGGCCGTGGCTCTCTTGT, (R) TGCACGTGACATCTAAGTTCTTTAGCA, (P) CAGCCTTCTGATTTCTGCAGCTCTGTGT; TNF- α : (F) CGGAACCAAGCTTAGAACTTTA, (R) CACTGTGCAGGCCACACATT, (P) ACCACCACTTCGAAACCTGGGATTCA; GM-CSF: (F) CCGCCTGGAGCTGTACAAG, (R) TAATCTGGGTTGCA-CAGGAAGTT, (P) CCCTTGACCATGATGGCCAGCC; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): (F) GAAGGTGAAGGTCGGAGTC, (R) GAAGATGGTGATGGGATTTC, (P) CAAGCTTCCCGTTCTCAGCC. These self-designed and custom synthesized oligonucleotide primers were obtained from Perkin Elmer (Wietarstadt, Germany) or Eurogentec (Seraing, Belgium). The TaqMan probes of the target genes (cytokines) were labeled with 6-carboxyfluorescein (FAM) as the reporter dye and 6-carboxytetramethylrhodamine (TAMRA) as the fluorescent quencher. The probe for the housekeeping gene (GAPDH) was labeled with VIC[®] (Applied Biosystems) as the reporter dye and TAMRA as fluorescent quencher. The PCR thermal cycle conditions were as follows: 50°C for 2 min, 95°C for 10 min, then 45 cycles at 95°C for 15 s and 60°C for 1 min. Quantification of gene expression was performed using the comparative threshold cycle (C_T) method (Sequence Detector User Bulletin 2; Applied Biosystems) and reported as the fold difference relative to the housekeeping gene. To calculate the fold change (increase or decrease), the

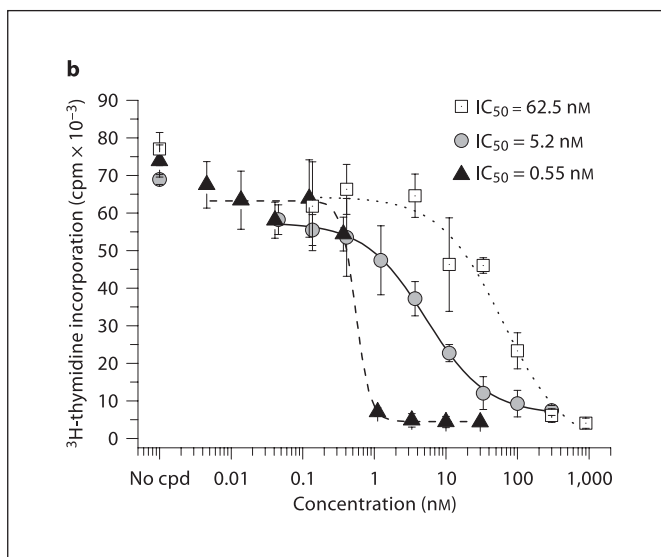
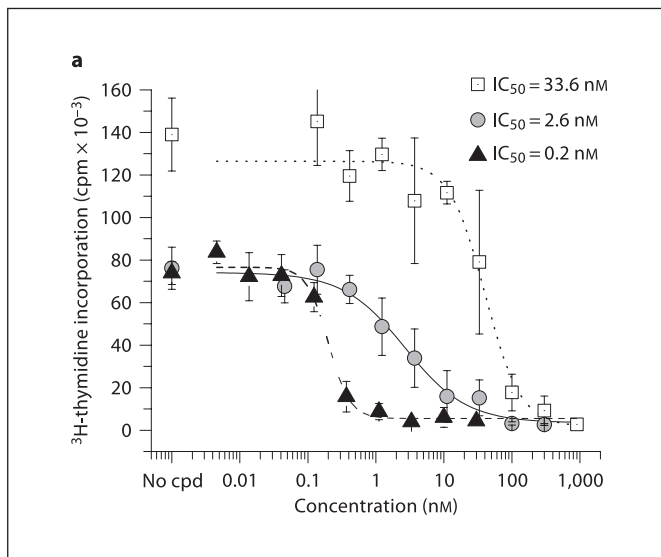


Fig. 1. Inhibition of alloantigen-stimulated polyclonal T cell proliferation by CNI. CD4⁺ T cells (**a**) or CD4⁺ plus CD8⁺ pan T cells (**b**) were subjected to stimulation by allogeneic DC (**a**) or SEB presented on autologous DC (**b**) in the absence and presence of different concentrations of CsA (squares), pimecrolimus (circles) or tacrolimus (triangles). The counts per minute (cpm) were plotted against the CNI concentration, and the IC₅₀ values were calculated by curve fitting in Origin®. Representative results obtained in 1 of 4 (**a**) and 1 of 5 (**b**) experiments are shown. cpd = Compound.

C_T of the housekeeping gene (GAPDH) was subtracted from the C_T of the target gene to yield the ΔC_T . Change in expression of the normalized target gene as a result of induction was expressed as $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T$ induced samples – C_T uninduced samples.

Table 1. Inhibition of T cell proliferation in MLR

Cells used	Assay	IC ₅₀ , nM			Pim/Tac ratio
		CsA	Pim	Tac	
CD4 ⁺	auto-MLR	41.2	5.39	0.645	8.4
Pan T	auto-MLR	12.2	1.46	0.34	4.3
Pan T	auto-MLR	51.8	4.99	0.565	8.8
CD4 ⁺	auto-MLR	62.5	5.2	0.55	9.5
Pan T	allo-MLR	n.d.	1.41	0.36	3.9
Pan T	allo-MLR	n.d.	2.1	0.18	11.7
Pan T	allo-MLR	n.d.	1.41	0.13	10.8
Pan T	allo-MLR	n.d.	2.7	0.63	4.3
CD4 ⁺	allo-MLR	33.6	2.6	0.2	13.0
Average		40.3	3.03	0.40	8.29
SD		19.1	1.70	0.20	3.41

Pim = Pimecrolimus; Tac = tacrolimus; Pan T = pan T cells; n.d. = not detectable.

Statistical Analysis

Student's paired t test was used for statistical evaluation of the significance of differences between logarithmically transformed IC₅₀ values using SigmaStat®.

Results

Inhibition of T Cell Proliferation by Pimecrolimus and Tacrolimus in Primary MLR

The effect of pimecrolimus, tacrolimus and CsA on the growth of pan T cells or purified CD4⁺ T cells obtained from healthy donors was tested after stimulation by allogeneic DC (allo-MLR) or SEB plus autologous DC (auto-MLR). Representative results for each MLR are shown in figure 1. Based on the comparison of average IC₅₀ values from 9 independent experiments using T cells from 5 different donors, tacrolimus was about 8-fold more potent than pimecrolimus and about 100-fold more potent than CsA (table 1). The difference between the IC₅₀ values obtained for tacrolimus and pimecrolimus was highly significant ($p < 0.001$).

Inhibition of Cytokine Production and Proliferation in PBMC and T Cells

To evaluate the potencies of pimecrolimus and tacrolimus to inhibit the synthesis of T-cell-derived cytokines such as IL-2, IL-4, IL-8, IFN- γ , TNF- α and GM-CSF, PBMC were stimulated by SEB, and cell-free supernatants were taken after 24 h for subsequent multiplex analysis. The results from a series of 2 independent experi-

Table 2. Inhibition of cytokine synthesis in SEB-stimulated PBMC

Experiment	Compound	IC ₅₀ , nM						
		IL-2	IL-4	IL-8	IFN- γ	TNF- α	GM-CSF	average
PBMC No. 1	Tac	0.069	0.23	0.28	0.14	0.14	0.36	0.20
	Pim	0.53	1.3	2.4	1	1.1	3.9	1.71
	CsA	6.1	19	18	10	9.9	30	15.50
	Pim/Tac	7.7	5.7	8.6	7.1	7.9	10.8	8.0
	CsA/Pim	11.5	14.6	7.5	10.0	9.0	7.7	10.1
PBMC No. 2	Tac	0.064	0.26	0.3	0.16	0.19	0.26	0.21
	Pim	0.55	2.1	3.3	2	1.7	2.8	2.08
	CsA	3.9	18	12	8.4	7.3	17	11.10
	Pim/Tac	8.6	8.1	11.0	12.5	8.9	10.8	10.0
	CsA/Pim	7.1	8.6	3.6	4.2	4.3	6.1	5.6

Tac = Tacrolimus; Pim = pimecrolimus.

Table 3. Inhibition of anti-CD3/CD28-stimulated cytokine synthesis in purified T cells

	Compound	IC ₅₀ , nM				
		GM-CSF	TNF- α	IFN- γ	IL-2	average
Experiment No. 1	Pim	19	3.4	2.37	0.79	6.39
	Tac	1.5	0.49	0.51	0.12	0.66
	Pim/Tac ratio	12.7	6.9	4.6	6.6	7.7
Experiment No. 2	Pim	6.11	2.13	3.19	0.35	2.95
	Tac	0.51	0.24	0.64	0.096	0.37
	Pim/Tac ratio	12.0	8.9	5.0	3.6	7.4
Experiment No. 3	Pim	2.08	1.15	1.01	0.51	1.19
	Tac	0.18	0.11	0.2	0.065	0.14
	Pim/Tac ratio	11.6	10.5	5.1	7.8	8.7

Pim = Pimecrolimus; Tac = tacrolimus.

ments are shown in table 2 and indicate that tacrolimus was about 8- to 10-fold more potent than pimecrolimus, which was about 5- to 10-fold more potent as compared with CsA. Similar results were obtained in another 3 independent experiments using purified pan T cells stimulated with anti-CD3 and anti-CD28 mAb (table 3), making a direct impact of the compounds on antigen-presenting cell (APC) functions unlikely.

Inhibition of Cytokine Expression in the Human T Cell Line Jurkat

The kinetics of cytokine gene expression and protein production was investigated in PMA plus PHA-stimu-

lated Jurkat cells. Total RNA was isolated from Jurkat cells at 0, 2, 6, 24 or 48 h after induction and analyzed using quantitative RT-PCR. The supernatants were collected at 24 and 48 h for determination of cytokine levels via ELISA. As shown in table 4, pimecrolimus and tacrolimus potently inhibited the expression of TNF- α , GM-CSF, IL-2, IL-3 and IL-8 both at the mRNA and at the protein level. The IC₅₀ values of pimecrolimus were found to be very similar to those obtained for tacrolimus, and in most cases, differed by less than a factor of 2. CsA consistently showed 10- to 20-fold higher IC₅₀ values as compared with pimecrolimus or tacrolimus.

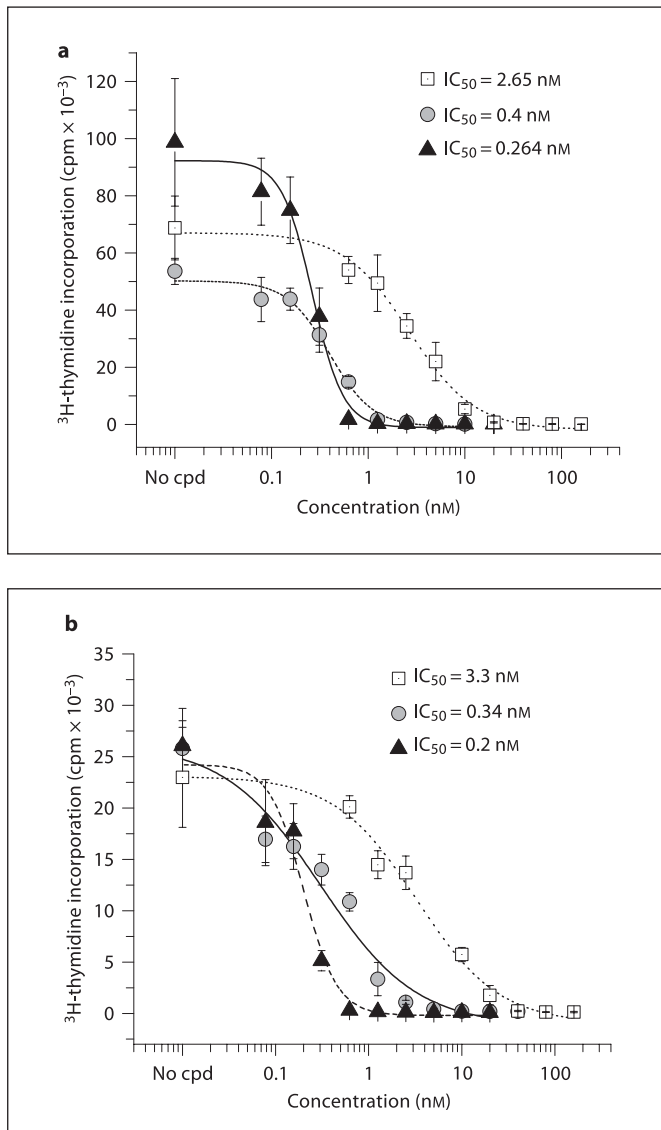


Fig. 2. Inhibition of the antigen-specific recall response of dust mite-specific T cell clones. Dust mite antigen-pulsed allogeneic, HLA-DP4-matched DC were used to stimulate the Th0 type clone 4:2.15 (**a**) or the Th2 type clone 4:3.1 (**b**) and incubated in the absence or presence of different concentrations of CsA (squares), pimecrolimus (circles) or tacrolimus (triangles). The counts per minute (cpm) were plotted against the CNI concentration and the IC_{50} values calculated by curve fitting in Origin®. Data shown were obtained in 1 of 3 experiments that yielded similar results. cpd = Compound.

Inhibition of TCC Antigen-Specific Proliferation

To assess the potency of the CNI to inhibit the allergen-specific proliferation, we used the TCC4:2.15 and TCC4:3.1, which were derived from skin punch biopsies

Table 4. Inhibition of cytokine mRNA and protein synthesis in Jurkat T cells

Cytokine	Substance	IC_{50} , nM			
		mRNA		protein	
		2 h	6 h	24 h	48 h
TNF- α	Pim	0.29	0.53	0.14	0.13
	Tac	0.33	0.42	0.09	0.09
	CsA	1.03	4.35	1.41	1.37
GM-CSF	Pim	0.32	0.83	0.18	0.12
	Tac	0.33	0.60	0.09	0.07
	CsA	2.51	3.05	1.94	3.83
IL-2	Pim	0.31	0.82	0.22	0.16
	Tac	0.31	0.34	0.10	0.09
	CsA	1.27	5.29	2.16	2.66
IL-3	Pim	0.63	0.58	0.11	0.07
	Tac	0.31	0.32	0.07	0.06
	CsA	3.37	2.83	0.78	0.88
IL-8	Pim	0.30	0.64	0.20	0.16
	Tac	0.31	0.31	0.08	0.10
	CsA	4.66	6.40	1.75	2.29

Pim = Pimecrolimus; Tac = tacrolimus. Stimulated control cytokine mRNA levels (2 h; 6 h) in fold induction relative to unstimulated levels: TNF- α (114; 24), GM-CSF (13,600; 239,000), IL-2 (108; 5,350), IL-3 (3.9; 417), IL-8 (2,620; 5,130). Stimulated control cytokine protein levels (24 h; 48 h) in nanograms/milliliter: TNF- α (0.70; 0.55), GM-CSF (1.1; 0.86), IL-2 (44; 41), IL-3 (13; 8.3), IL-8 (0.14; 0.088).

of a house dust mite-allergic patient [13]. DC preselected for matching the HLA class II restriction element were pulsed with *Dpt* and cultured together with TCC in the absence or presence of graded amounts of pimecrolimus, tacrolimus or CsA. As shown in figure 2, pimecrolimus and tacrolimus similarly inhibited TCC proliferation, their IC_{50} values differing by a factor of less than 2. In contrast, CsA was about 10- to 20-fold less active than either tacrolimus or pimecrolimus.

Inhibition of Cytokine Synthesis following Antigen-Specific or Non-Specific TCC Stimulation

Next, we investigated whether the secretion of Th1 cytokines (IFN- γ and IL-2), Th2 cytokines (IL-4, IL-5) or non-subset restricted cytokines such as IL-10, TNF- α and GM-CSF were inhibited to a comparable degree by pimecrolimus and tacrolimus. Stimulation performed in the absence of compounds revealed that TCC4:3.1 can be

Table 5. IC₅₀ values (nM) and ratios for inhibition of cytokine synthesis by skin-derived TCC stimulated by antigen-pulsed DC

Antigen-pulsed DC		TCC 4:2.15			TCC 4:2.20			TCC 4:3.1		
pattern	cytokine	Pim	Tac	Pim/Tac ratio	Pim	Tac	Pim/Tac ratio	Pim	Tac	Pim/Tac ratio
Th1 type	IL-2	n.d.	n.d.	n.a.	n.d.	n.d.	n.a.	1.13	0.84	1.34
	IFN- γ	1.11	0.494	2.25	0.477	0.4	1.19	1.24	0.94	1.32
Th2 type	IL-4	0.629	0.361	1.74	n.d.	n.d.	n.a.	1.21	0.63	1.92
	IL-5	1.88	1.031	1.82	n.d.	n.d.	n.a.	2.05	1.14	1.80
Th1 and Th2	IL10	0.845	0.431	1.96	0.699	0.514	1.36	0.76	0.72	1.05
	TNF- α	1.51	0.878	1.72	1.356	0.635	2.14	1.66	1.03	1.61
	GM-CSF	3.2	1.187	2.70	1.119	0.496	2.26	4.54	1.17	3.88
Average		2.03			1.74			1.84		

The culture supernatants were harvested 48 h after stimulation and assayed by the Luminex technology. Pim = Pimecrolimus; Tac = tacrolimus; n.d. = not detectable; n.a. = not applicable.

Table 6. IC₅₀ values (nM) and ratios for inhibition of cytokine synthesis of anti-CD3/PMA-stimulated skin-derived TCC

Anti-CD3/PMA		TCC 4:2.15			TCC 4:2.20			TCC 4:3.1		
pattern	cytokine	Pim	Tac	Pim/Tac ratio	Pim	Tac	Pim/Tac ratio	Pim	Tac	Pim/Tac ratio
Th1 type	IL-2	0.515	0.380	1.36	0.447	0.367	1.22	n.d.	n.d.	n.a.
	IFN- γ	0.915	0.492	1.86	1.143	0.57	2.01	n.d.	n.d.	n.a.
Th2 type	IL-4	0.71	0.405	1.75	0.537	0.403	1.33	n.d.	n.d.	n.a.
	IL-5	0.721	0.446	1.62	0.839	0.568	1.48	0.303	0.222	1.36
Th1 and Th2	IL-10	0.6	0.44	1.36	0.552	0.411	1.34	0.176	0.148	1.19
	TNF- α	0.887	0.479	1.85	0.883	0.506	1.75	0.859	0.591	1.45
	GM-CSF	0.897	0.489	1.83	0.958	0.541	1.77	0.545	0.355	1.54
Average		1.66			1.56			1.39		

The culture supernatants were harvested 48 h after stimulation and assayed by the Luminex technology. Pim = Pimecrolimus; Tac = tacrolimus; n.d. = not detectable; n.a. = not applicable.

regarded as a Th2 clone due to its very low production of IL-2 and IFN- γ and very high secretion of IL-5 and moderate production of IL-4 when stimulated by *Dpt*-presenting DC. The TCC4:2.15 and TCC4:2.20 showed a Th0 profile secreting IL-2, IFN- γ , as well as IL-4 and IL-5 after antigen-specific or anti-CD3 plus PMA-mediated stimulation (data not shown). Table 5 shows that the individual IC₅₀ values for inhibition of cytokine production induced by antigen-pulsed DC differed by a factor of less than 2, and similar IC₅₀ values and ratios were obtained for anti-CD3 plus PMA-stimulated TCC (table 6).

Comparison of Pimecrolimus and Tacrolimus in Primary and Secondary T Cell Stimulation

The observation that tacrolimus had a higher potency than pimecrolimus to inhibit primary stimulation of T cells but was similarly active on antigen recall and anti-CD3 antibody-mediated responses of TCC prompted us to investigate the activity profile of both CNI on T cells subjected to a primary and secondary activation. Parallel to the primary evaluation of growth inhibition by pimecrolimus and tacrolimus, T cells were expanded in cocultures with DC for 11 days and then subjected to a

Table 7. Inhibition of T cell proliferation in primary and secondary MLR

Donor	Stimulation	Primary assay			Secondary assay		
		IC ₅₀ , nM		Pim/Tac ratio	IC ₅₀ , nM		Pim/Tac ratio
		Pim	Tac		Pim	Tac	
HV	S-antigen	1.07	0.16	6.7	0.11	0.13	0.8
HV	S-antigen	1.47	0.42	3.5	0.13	0.11	1.2
HV	allo-MLR	1.15	0.34	3.4	0.184	0.18	1.0
HV	allo-MLR	1.2	0.24	5.0	0.12	0.18	0.7
RC	S-antigen	1.37	0.37	3.7	0.32	0.41	0.78
AD	S-antigen	3.78	0.33	11.5	0.014	0.095	0.15
RC	S-antigen	9.2	0.88	10.5	0.2	0.13	1.54
AD	S-antigen	1.34	0.094	14.3	0.23	0.18	1.28
Average		2.57	0.35	7.3	0.2	0.2	0.9

Pim = Pimecrolimus; Tac = tacrolimus; HV = healthy volunteers; RC = rhinoconjunctivitis patients; AD = atopic dermatitis patients.

secondary stimulation using conditions identical to those applied in the primary assay using freshly thawed allogeneic or autologous SEB-loaded DC. In this series of experiments, pan T cells or CD4+ T cells purified from the PBMC of 4 different healthy subjects and 4 different allergic donors were used. The comparison of the IC₅₀ values obtained in primary MLR (table 7) confirmed that pimecrolimus was consistently and significantly ($p < 0.001$) less potent than tacrolimus to inhibit the primary T-cell proliferation, yielding about 7-fold higher IC₅₀ values. However, in secondary assays, tacrolimus and pimecrolimus showed equivalent inhibitory potencies. CD45 isoform analysis by FACS showed that at least 50% of the T cells expressed CD45RA at the start of the experiment, whereas more than 95% of all cells were positive for CD45RO after 11 days of culture, when they were used for secondary stimulation (data not shown).

Discussion

The recent observation of a differential profile of systemically applied pimecrolimus and tacrolimus in CHS [11] and the paucity of published data on a side-by-side comparison of the two drugs in vitro led us to investigate their potency in the context of primary and secondary T cell stimulations. Here, we show that pimecrolimus was consistently less active than tacrolimus on freshly isolated PBMC, purified CD4+ T cells or pan T cells containing both CD4+ plus CD8+ T cell subsets. This distinction

was apparent, irrespective of whether primary T cell activation was mediated by allogeneic DC, by the bacterial superantigen SEB using autologous DC or PBMC, or by APC-independent stimulation with antibodies against the T cell receptor complex CD3 and CD28. Overall, the difference in potency between pimecrolimus and tacrolimus ranged between 8-fold for the inhibition of T cell proliferation obtained in the allogeneic or autologous MLR and 10-fold for the inhibition of cytokine production by SEB- or antibody-stimulated T cells. The IC₅₀ values obtained for the inhibition of proliferation or cytokine release by pimecrolimus and tacrolimus differed highly significantly from each other ($p < 0.001$) using cells derived from 7 different donors. In the MLR and the cytokine induction experiments, CsA was about 50- to 100-fold less potent than tacrolimus and about 10-fold less potent as compared with pimecrolimus. These results are in line with the different potencies of tacrolimus and CsA previously reported [6, 7, 15] and consistent with the reported 10-fold difference in potencies between CsA and pimecrolimus [9, 10].

Since a preliminary report of the inhibition of Jurkat T cell activation showed similar potencies of tacrolimus and pimecrolimus [16], it was of interest to investigate all three CNIs side by side for the ability to inhibit the activation-induced cytokine transcription and protein synthesis. The data obtained for the inhibition of cytokine mRNA induction and protein release indicated a 2-fold difference of IC₅₀ values for pimecrolimus and tacrolimus, which contrasts with a significantly higher potency of tacrolimus to

inhibit primary T cell activation. Therefore, we extended the analysis to house dust mite antigen-specific TCC that were derived from skin biopsies of an atopic dermatitis patient and described previously [12, 13]. Restimulation of three TCC revealed that pimecrolimus or tacrolimus had very similar potencies to inhibit the proliferation and cytokine synthesis, regardless of whether the T cells were stimulated by anti-CD3 plus PMA or specific antigen presented on HLA class II matched DC. This argues against a direct influence on costimulatory pathways or APC functions by the compounds. Rather, our findings strengthen the hypothesis that the sensitivity of T cells towards pimecrolimus increases both absolutely and relative to tacrolimus if they had been activated and stimulated to proliferate previously. To substantiate this idea, T cells purified from PBMC of 4 healthy and 4 allergic donors were stimulated in the primary allogeneic and autologous MLR, expanded in culture, and then subjected to a secondary stimulation. Pimecrolimus was observed to be about 7-fold less potent than tacrolimus as judged by a comparison of the average IC_{50} values (table 5). Statistical evaluation confirmed that the decrease in IC_{50} values seen for pimecrolimus in secondary MLR was significant ($p < 0.001$), whereas the differences of IC_{50} values between primary and secondary MLR obtained for tacrolimus were not significant. This indicates an enhanced sensitivity of prestimulated T cells towards inhibition by pimecrolimus but not tacrolimus. In this context, it is worth noting that the question of a differential sensitivity of naïve versus memory-type T cells towards CsA has remained unresolved. Although one study showed the CD4+/CD45RO+ T cell subset to be relatively more sensitive than the CD45RA+ subset towards inhibition by CsA in vitro [17], a more recent publication [18] showed exactly the opposite and challenges the conclusions of the former. The data presented here support the conclusion of the former study [17], since a higher sensitivity towards inhibition by CsA was observed in Jurkat cells and TCC (table 3; fig. 2) when compared with primary T cells (fig. 1; tables 1, 2).

What may be the clinical relevance of the differential inhibition of primary and secondary T cell responses by pimecrolimus and tacrolimus? Antigen-specific effector/memory T cells dominate the dermal and epidermal T cell infiltrate in lesional skin of atopic eczema [19] and are considered to be relevant targets of topically applied CNI in the context of atopic dermatitis [20, 21]. Moreover, antigen-specific TCC can be long-lived in atopic dermatitis patients. Notably, the house dust mite-specific TCC 4:3.1 used in the present study could be recovered from the antigen-challenged skin sites of the same atopic der-

matitis patient several years after their primary isolation [22]. As shown in the present study, the T-cell-receptor-mediated and antigen-specific recall stimulation of the TCC 4:3.1 was similarly inhibited by pimecrolimus and tacrolimus in vitro, in line with similar inhibition of secondary polyclonal T cell proliferation by both CNI. On the other hand, the consistently lower sensitivity of primary activated, PBMC-derived T cells towards pimecrolimus indicates a lower ability to interfere with de novo T cell responses to antigen. This assumption is supported by a recent study showing that topical pimecrolimus applied for 3 weeks reduced the inflammatory cell infiltrate and T cell cytokine expression in the dermis, whereas it did not reduce the capacity of PBMC-derived T cells to proliferate or secrete cytokines following PHA stimulation ex vivo [23]. This picture fits with the differential profile of systemically applied pimecrolimus and tacrolimus shown in CHS. Although both drugs equipotently inhibited the inflammatory reaction upon challenge with the allergen, only tacrolimus inhibited the priming responses when administered during the sensitization phase [11]. These in vivo results indicated that pimecrolimus has a selectivity for primed effector T cells not seen with tacrolimus since the lymph node concentration of the drugs would be relevant for inhibition of naïve T cell priming by skin-draining DC, whereas the CNI concentration in dermis and epidermis should be decisive for suppression of the local inflammatory response governed by effector/memory T cells. In support of this concept, clinical studies for up to 3 months with oral pimecrolimus in psoriasis [24, 25] and atopic dermatitis [26] patients indicated therapeutic efficacy and a lack of systemic immunosuppression [24], as well as of 'calcineurin-type' side effects such as kidney toxicity and hypertension. This evidence for an improved therapeutic window may be explained by the differential expression of drug-binding or signal-transducing molecules, such as macrophilin-12 or calcineurin A and B subunits and NFAT, and is subject to further analysis.

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