

Original Article

Modulation of NFAT-5, an outlying member of the NFAT family, in human keratinocytes and skin

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Abstract: *Background:* Cyclosporin A (CsA) and tacrolimus block T cell activation by inhibiting the phosphatase calcineurin and preventing translocation from the cytoplasm to the nucleus of the transcription factor Nuclear Factor of Activated T cells (NFAT). NFAT compose a family of transcription factors that are turned on during T cell activation. *Aims:* To study the expression of NFAT-5 mRNA and protein in normal human keratinocytes and to investigate the cellular and subcellular pattern of expression of NFAT-5 in normal human skin and psoriasis, and analyze effects of different agonists and ultraviolet radiation on NFAT-5 in normal human skin. *Methods:* Tissue cultures, Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), Western analysis, immunostaining, confocal microscopy. *Results:* Sequencing of RT-PCR products confirmed the identity of the product that showed 100 % homology with the predicted NFAT-5 sequence. anti-NFAT-5 mainly detected a single band in cultured keratinocytes and dermal fibroblasts using Western analysis. Immunohistochemistry showed that epidermal keratinocytes and dermal fibroblasts in normal human and psoriatic skin express NFAT-5. NFAT-5 showed predominantly nuclear localization in epidermal keratinocytes and dermal fibroblasts within five normal adult skin biopsies. Our data also suggest that UV irradiation reduces NFAT-5 nuclear localization within the epidermis. Unlike NFAT 1-4, NFAT-5/TonEBP was localized to both nucleus and cytoplasm of cultured keratinocytes. Cyclosporin A induces nuclear membrane translocation of NFAT-5 in cultured keratinocytes and raffinose (a hypertonicity inducing agent) induces more nuclear localization of NFAT-5 compared to untreated cells. In addition, differentiation-promoting agonists that induce sustained rise in intracellular calcium did not result in changes in NFAT-5 localization in cultured keratinocytes. *Conclusion:* These studies provide the first observation of expression of NFAT-5/TonEBP mRNA protein in cultured keratinocytes and dermal fibroblasts and possible functional regulation in cultured keratinocytes. CsA and raffinose effects on NFAT-5/TonEBP in cultured keratinocytes suggest diverse intracellular signaling pathways for NFAT-5/TonEBP in these cells, and that NFAT-5/TonEBP might function to translate different extracellular stimuli into appropriate functional responses.

Key Words: Cyclosporin A (CsA), Human keratinocytes, Hypertonicity, NFAT-5 (TonEBP), psoriasis, UVR

Introduction

Cyclosporin A (CsA) is widely utilized for the treatment of inflammatory skin diseases such as psoriasis. The therapeutic effects of CsA are thought to be mediated via its immunosuppressive action on infiltrating lymphocytes in skin lesions. CsA and tacrolimus block T cell activation by inhibiting the phosphatase calcineurin and preventing translocation from the cytoplasm to the nucleus of the transcription factor Nuclear

Factor of Activated T cells (NFAT). NFAT compose a family of transcription factors that are turned on during T cell activation (**Figure 1**). Five different members of the NFAT family of transcription factors have been identified so far. The NFAT family is composed of five members: NFAT 1 to 5 [1]. NFAT-5, the most recent addition to the NFAT/Rel family of transcription factors, was isolated by five independent laboratories, on the basis of its high degree of sequence similarity (41%-45%) to the DNA-binding domain (Rel homology

region of NFAT), who named it NFAT-5 [2], NFATz [3], Osmotic Response Element Binding Protein (OREBP) [4] and NFATL1 [5] respectively. In addition, NFAT-5 was independently cloned in a yeast one-hybrid assay as Tonicity-responsive Enhancer Binding Protein (TonEBP), a Rel-like protein that stimulates transcription in response to hypertonicity [6].

Isolation of full-length cDNA encoding NFAT-5 showed that this new Rel-like protein possesses distinct features from those of NFAT 1-4. First, it lacks the majority of Fos and Jun contact residues and does not bind co-

operatively with Fos/Jun to DNA. Second, NFAT-5 lacks the conserved regulatory domains of the calcineurin-regulated NFATs. Furthermore, immunocytochemical and biochemical analysis showed that NFAT-5 is localized to the nucleus and the cytoplasm in different cells and neither its subcellular distribution nor its phosphorylation state are affected by calcineurin [2]. In addition, NFAT-5 resembles an NFκB/Rel family stable dimer in solution in the absence of DNA, and dimerization is obligatory for DNA binding and transcriptional activity. Lopez-Rodriguez et al. showed that NFAT-5/TonEBP uniquely links the NFAT and NFκB/Rel families and regulates

the production of specific cytokines in lymphocytes stimulated by osmotic stress [7]. Thus, NFAT-5 is the only member of the Rel/NFAT family that is activated by hypertonic stimulation, regulating not only the expression of osmoregulatory genes but also TNFα and lymphotoxin-β gene transcription in osmotically stressed cells [7]. These features support the hypothesis that NFAT-5 is an outlying member of the NFAT family. However, Trama et al. have demonstrated that NFAT-5 can be induced in both primary T lymphocytes and differentiated Th1 and Th2 cells upon mitogen or antigen receptor-dependent activation and this induction was inhibited by CsA and tacrolimus [5]. In addition, a reporter gene analysis showed that T-cell receptor cross-linking resulted in the induction of NFAT-5 in human Jurkat cells [5]. This study demonstrated that the induction of NFAT-5/TonEBP, in response to receptor-mediated mitogenic signals is dependent upon calcineurin activation, as this induction was inhibited by CsA and tacrolimus, while the induction by hyperosmotic stimuli was not [5].

Hypertonicity is stressful to the cells of virtually all organisms [8-11]. As cells are permeable to water, exposure to a hypertonic environment causes the cells to shrink and elevates the concen-

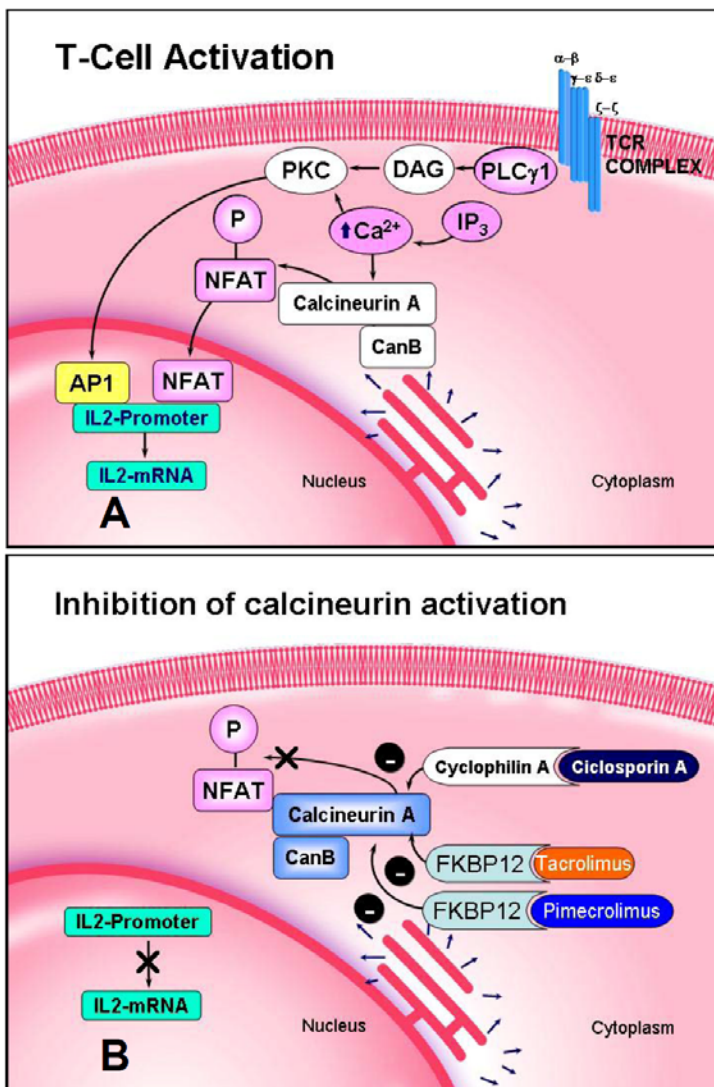


Figure 1. Schematic representation of T cell activation (A) and mechanism of action of cyclosporin A (CsA), tacrolimus and pimecrolimus (B). Inhibition of the phosphatase calcineurin blocks nuclear translocation of NFAT.

tration of intracellular ions. Mammalian cells respond to hypertonic conditions by accumulating small organic molecules known as compatible osmolytes [8]. Compatible osmolytes are small organic solutes comprising five members: sorbitol, myo-inositol, betaine, taurine and glycerophosphocholine [11]. Myo-inositol, betaine, and taurine are taken up by sodium and sodium/chloride-dependent transporters. Sorbitol is synthesised from glucose catalyzed by aldose reductase. Glycerophosphocholine is made from phosphatidylcholine [12, 13]. All compatible osmolytes are transcriptionally regulated [11, 14]. Unlike with bacteria and yeast, little is known how mammalian cells recognise hypertonicity and how the signal is conveyed to the regulatory sequence element named TonEBP [6, 15]. It has been suggested that the presence of these compounds balances increased extracellular tonicity and thus protects the cell from the damaging effects of elevated intracellular electrolyte concentrations [6, 9, 10, 15]. Miyakawa et al. reported the cloning and characterisation of the first animal transcription factor (NFAT-5/TonEBP) responsible for regulating osmolyte transporter genes during osmotic stress [6, 15]. Transcription of genes stimulated by hypertonicity plays a critical role in adaptation of mammalian cells to hypertonicity [9-11]. NFAT-5/TonEBP is known to mediate cellular responses to osmotic stress [2, 6, 7, 16]. For example, studies in Madin-Darby canine Kidney (MDCK) cells have shown that NFAT-5/TonEBP localizes to the cytoplasm and nucleus in isotonic conditions and gradually shifts to a predominantly nuclear location when cells are cultured in hypertonic medium [6]. This redistribution occurs over the course of hours [6].

NFAT-5/TonEBP is evolutionary the oldest member of the NFAT/Rel family of transcription factors. The single NFAT-like protein encoded in the *Drosophila* genome is closely related to NFAT-5 [17]. Recently, human NFAT-5 gene has been mapped to chromosome 16 by PCR using DNA from hybrid cell lines. The exact position of the human gene was reported to be between D16S496 and W15254 within the 16q22.1 sub-band. The murine gene has been localized to chromosome 8D [18, 19].

The first aim of this study was to investigate the expression of NFAT-5 mRNA in cultured

keratinocytes and cultured dermal fibroblasts. As a step towards elucidating the function of NFAT-5/TonEBP in skin, the subcellular localization of NFAT-5/TonEBP in response to selected agonists in cultured human keratinocytes was investigated. Recently, the expression of different genes induced by osmotic stress in human oral keratinocytes has been described [20]. Therefore, the activation of NFAT-5/TonEBP pathways in cultured human keratinocytes by hypertonicity was examined. Human keratinocytes were shown to respond to osmotic stress by the induction of heat shock proteins through a p38 MAPK regulated mechanism [21]. UV-induced phosphorylation of p38 in cultured keratinocytes has been described [22]. In addition, UV light induces phosphorylation of c-Jun through JNK [23] and JNK bound to c-Jun may phosphorylate NFAT, the binding site of which resides next to AP-1 sites [24]. Furthermore, hypertonicity also induces the expression of tumor suppressor p53 and activates p53 by phosphorylation of serine 15 [25]. Forced downregulation of p53 results in apoptosis suggesting that p53 activation in response to hypertonicity are very similar to those of ionizing radiation that causes DNA double-strand breaks [25]. Therefore, the effects of both UVA and UVB irradiation on NFAT-5/TonEBP localization in normal adult human skin were also studied using immunohistochemical techniques.

At least two signals are required for T cell activation. Phorbol ester and calcium ionophore (ionomycin) or co-stimulation of both TCR and CD28 generates these two signals. The activation of JNK was shown to be dependent on both signals [26]. These results are consistent with previous reports showing that combination of calcineurin activation and protein kinase C (PKC) resulted in JNK activation [27]. Recently, the immunosuppressive effects of CsA and tacrolimus were also shown to be mediated through inhibition of both JNK and p38 pathways activation [26, 28-30]. These inhibitory effects are mediated through the CsA-cyclophilin and tacrolimus-FKBP12 complexes [28, 29]. These results may indicate that the immunosuppressive effects on both CsA and tacrolimus are attributed, at least in part, to inhibition of JNK and p38 pathways. In addition, CsA and tacrolimus do not block stress-induced activation of JNK/p38 pathways [28, 29]. Matsuda et al. also

reported that dominant negative mutants that block JNK/p38 pathways abolish NFAT transcriptional activity [28]. Therefore, two distinct pathways, the calcineurin/NFAT pathway and JNK/p38 pathway, appear to be targeted by CsA and tacrolimus to inhibit T cell activation [29]. These data support previous reports that showed that CsA and tacrolimus can inhibit an antigen-specific signaling in a calcium/calcineurin independent manner in a T cell line [31, 32]. In summary, IL-2 production is inhibited by CsA and tacrolimus via inhibition of two major pathways: 1) the calcineurin/NFAT and 2) JNK/p38 pathways. CsA and tacrolimus effects on NFAT-5/TonEBP were also investigated in cultured keratinocytes (Materials and Methods).

Materials and Methods

Cyclosporin A (CsA) and tacrolimus were provided by Novartis Pharma AG, (Basil, Switzerland) and Fujisawa Pharmaceutical Co (Osaka, Japan), respectively. Tacrolimus was also obtained from Affinity Research Products Ltd (Exeter, UK). Raffinose was obtained from Sigma laboratories (Poole, UK). Keratinocytes growth medium (MCDB 153) and trypsin/ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma laboratories (Poole, UK). NFAT-5 primers were synthesised by MWG-Biotech AG (Ebersberg, Germany). Keratinocytes differentiation agents and growth factors including TPA, ionomycin, and Dimethyl Sulphoxide (DMSO) (vehicle control) were obtained from Sigma (Poole, UK). Precast polyacrylamide gels were purchased from Invitrogen (Paisley, UK). Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes, ECL molecular weight markers were obtained from Amersham (Buckinghamshire, UK). Prestained protein standards were provided by Bio-Rad Laboratories Ltd (Herts, UK). Anti-NFAT-5 was obtained from Santa Cruz Biotechnology Inc., CA, USA.

Tissue culture

The general tissue culture methods used followed those described by Freshney [33]. Keratinocytes were isolated from normal human skin obtained from plastic and surgical procedures. Keratinocytes were cultured in T75 flasks in MCDB-153 (Sigma, Poole, UK) as described before [34, 35] The culture medium used for growing keratinocytes was the serum-free medium MCDB153 described

by Boyce and Ham [36], with modifications described by Wille and Pittelkow [37, 38]. Antibiotics were added to give a final concentration of penicillin G (5 IU/ml) and streptomycin (5 µg/ml) (Sigma; Poole, UK).

Immunofluorescence of cultured cells

1. Coverslip preparations: Cells were trypsinised from flasks and seeded onto sterile coverslips placed in twelve well plates, so that there were 3x10⁴ cells on each coverslip. Coverslips were incubated in an incubator at 37°C in 5% CO₂. Coverslips were prepared as described [39,40]. Keratinocytes or fibroblasts were treated with specific agents, DMSO (1:1000) (vehicle control), or switched to medium containing raised extracellular calcium (1.5 mM CaCl₂) 15 min and 18 h. Some coverslip cultures were pre-treated with CsA or tacrolimus for 1 h. After the time of incubations, the medium was aspirated and the cells were washed three times in Ca²⁺ and Mg²⁺-free ice cold PBS before being fixed.

2. Fixation method: The effects of permeabilisation and fixation conditions on the subcellular localization of antigens [41] was examined carefully. Fixation methods fall generally into two categories, organic solvents and cross-linking reagents. The optimal fixation method was chosen empirically [42].

3. Cell staining for immunofluorescence microscopy: Non-specific binding was blocked by incubating coverslips in blocking serum (diluted 1:60 in PBS) by using serum from the species in which the secondary antibody was raised [43, 44] for 10 min. 100 µl of primary antibodies against NFAT-5 was added to each coverslip and incubated at room temperature for 45 min. Cells were washed three times in PBS. Cells were then incubated with 100 µl of FITC-conjugated anti-rabbit and FITC-conjugated anti-goat secondary antibody for 45 min at room temperature. Cells were washed three times with Ca²⁺ and Mg²⁺-free PBS. Cells were then incubated with 50 µg/ml propidium iodide (PI) (Sigma Laboratories; Poole, UK) for 1 h at room temperature. Finally, cells were washed three times with Ca²⁺ and Mg²⁺-free PBS. Coverslips were mounted onto slides using vectorshield fluorescence mounting medium (Vector Laboratories Ltd; Peterborough, UK) and the edges sealed with clear nail varnish. Cells

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were stained with 2 µg/ml goat polyclonal anti-NFAT-5/TonEBP antibody. For negative control studies, goat IgG (2 µg/ml) was used. Cells were visualised using a Biorad confocal microscope.

4. Confocal microscopy: Cells were analyzed using a Bio-Rad MRC 600 confocal laser scanning microscope (BioRad; Herts, UK), mounted on a Nikon Optiphot II (Nikon UK Ltd; Surrey, UK) upright stand with a Krypton/argon laser giving 448 nm, 568 nm, and 647 nm excitation lines. Briefly, cells were fixed in 4% paraformaldehyde, permeabilised with 0.2% Triton X-100, incubated sequentially with goat-polyclonal anti-NFAT-5 antibody (2 µg/ml) or goat IgG (2 µg/ml), rabbit anti-goat FITC, propidium iodide (50 µg/ml) and visualized using a Biorad confocal microscope.

Reverse transcription-polymerase chain reaction

NFAT-5 cDNA sequences were obtained from GenBank at <http://www.ncbi.nlm.nih> and complementary primers were designed to amplify target sequence specific for NFAT-5. Primers sequences were confirmed using the blast analysis at <http://www.ncbi.nlm.nih.gov/blast>. Coding sequence for NFAT-5 was aligned using Lasergene software (DNA Star Inc., Madison; USA) and primers were designed for each calcineurin subtype or NFAT isoform in areas of low homology. Primer set for human NFAT 2 was forward: 5'CCACTCATACCAAGCAGTATG 3' and backward: 5'CCTGCTGCAATAGTGCATC 3', resulting in amplification of 340 bp.

1. Prevention of ribonuclease (RNases) contamination: RNases are particularly stable and thus difficult to destroy. A number of precautions were taken to avoid RNase contamination [45].

2. Isolation of RNA: Cultured keratinocytes and fibroblasts at approximately 70% confluence were washed twice with sterile Ca²⁺ and Mg²⁺-free PBS. Keratinocytes and fibroblasts were removed from flasks by treatment with 0.05% trypsin and 0.02% EDTA. Jurkat T cells (used as a positive control) grow in suspension and can be aspirated from flasks. Total RNA was isolated using RNeasy Mini Kit (QIAGEN; West Sussex, UK) according to manufacturer's instruction.

3. Polymerase Chain Reaction (PCR): 3-5 µl of cDNA was amplified in 50µl PCR reaction which consisted of 1.5 µl of 50 mM MgCl₂ (Bioline; London, UK), 5 µl 10x NH₄ buffer (Bioline; London, UK), 5 µl DMSO, 1.25 µl of 25 pmol forward primer, 1.25 µl of 25 pmol reverse primer and 4 µl of dNTP's (2.5 mM each dNTP). Distilled water was added to make the total reaction volume equal 50 µl. Negative controls were included in each reaction by replacing the cDNA with water. 0.2 µl of 0.625 U BioTaq™ DNA polymerase (Bioline; London, UK) was added to the reaction after heating to 94°C for 5 min, followed by 34 cycles of denaturation at 94°C for 1 min, re-annealing at 55-57°C for 1 min and elongation at 72°C for 2 min. A final cycle of 72°C for 15 min was used. Similar cycle conditions were used for each set of primers.

4. Agarose Gel Electrophoresis: PCR products were electrophoresed through 1.5% agarose gels to determine product size. Loaded samples were visualised on a UVP transilluminator and photographed (Mitsubishi camera/ Polaroid black and white film type 667).

5. Gel extraction: PCR products were gel purified using a QIAGEN kit (QIAGEN; West Sussex, UK) to obtain single fragments for sequencing. DNA was separated using agarose gel electrophoresis. The appropriate band was excised, weighed and sent for sequencing.

6. Sequencing of PCR products: Automated sequencing was carried out by MWG-Biotech AG (Ebersberg, Germany).

Western Blotting

Cells were lysed in 2 X Sodium Dodecyl Sulphate (SDS), sample buffer (125 mM Tris-HCl, pH 6.8, 0.05% bromophenyl blue, 4% SDS, 20% glycerol and 10% β-mercaptoethanol). Equal amounts of samples and enhanced chemiluminescence molecular weight markers (Amersham, Bucks, UK) were electrophoresed through 10% polyacrylamide gels, and Western Blotting were performed as described [35], using goat polyclonal anti-NFAT-5 antibody (0.4 µg/ml).

Subjects and immunohistochemical analysis of skin biopsies

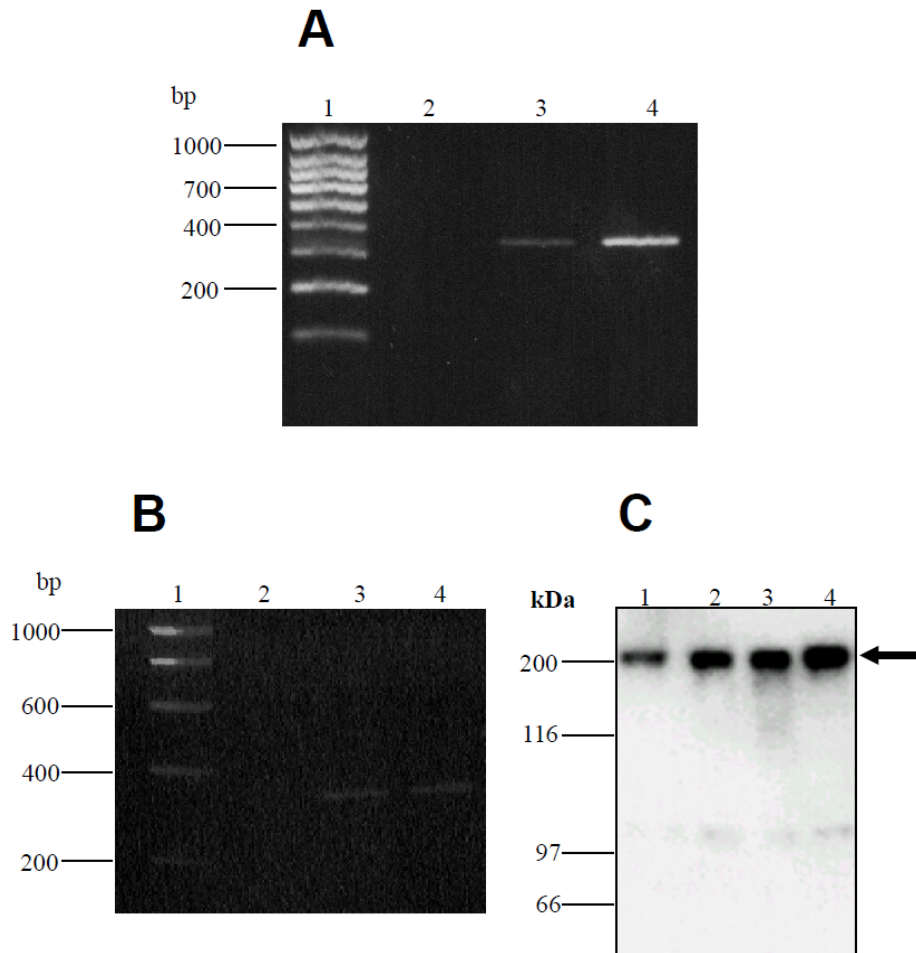


Figure 2. NFAT-5 mRNA and protein expression in cultured human keratinocytes and dermal fibroblasts. Total RNA was extracted from cultured cells, reverse transcribed and PCR performed with NFAT-5-specific primers. Reaction products were separated by electrophoresis in 1.5% agarose gels. (A), lane 1 hyperladder IV; lane 2, negative control (water); lane 3, Jurkat T cells; lane 4, cultured keratinocytes. (B), lane 1, hyperladder I; lane 2, negative control (water); lane 3, cultured keratinocytes; lane 4, cultured fibroblasts. Sequencing studies confirmed the expression of NFAT-5 in cultured cells. The predicted size of NFAT-5 is 340 bp. (C), cell lysates were prepared from cultured keratinocytes and dermal fibroblasts, separated by SDS-PAGE and immunoblotted with anti-NFAT-5 antibody. This experiment confirmed that the antibody used in immunostaining detects the appropriate molecular weight of NFAT-5 (202 kDa) (arrow). Lane 1, medium control (keratinocytes) (donor 1); lane 2, medium control (keratinocytes) (donor 2); lane 3, medium control (fibroblasts) (donor 1); lane 4, medium control (fibroblasts) (donor 2).

Five normal volunteers (3 male, 2 females, mean age 37 years) and 5 patients with stable plaque psoriasis (3 male, 2 females, mean age 50.5 years) were recruited for study as described [39]. Five μm frozen sections were stained with goat polyclonal anti-NFAT-5 (2 $\mu\text{g}/\text{ml}$) (Santa Cruz Biotechnology Inc., CA, USA). Equal concentration of goat IgG was used as a negative control. Sections were developed and assessed as described [39].

Subjects and administration of UVR

Investigations were carried out on the healthy skin of the lower back of four adult volunteers (three males, one female; mean age 36 years). Exclusion criteria included a past history of photosensitivity and any regular oral medication. Seven doses of UVB (10-80 mJ/cm^2 , $\sqrt{2}$ increments) and five doses of UVA (10-40 J/cm^2 , $\sqrt{2}$ increments) were

administered separately to the lower back of 4 volunteers. At 24 h post-irradiation, erythema intensities were recorded in triplicates using a reflectance instrument (Diastorn; Andover, UK) and a visual assessment of the minimal erythema dose (MED) was made [46]. Under local anaesthesia (intradermal lignocaine), 4 mm punch biopsies were taken from UVA and UVB irradiated sites of equal erythema that approximated to moderate erythema together with unirradiated control skin and subsequently used for immunostaining of skin biopsies. This work was carried out with Dr. M. Murphy and Dr. P. Farr.

1. Sources of UV irradiation: For irradiation of human skin, the source of both UVA and UVB was an irradiation monochromator (Applied Photophysics Limited, Surrey, UK) [Model UV90] fitted with a 10 mm diameter liquid-filled light guide, optically coupled to a light exposure Xenon arc lamp. The central wavelength and band was 300 ± 5 nm for UVB and 350 ± 30 nm for UVA in conjunction with a Schott WG355 filter for UVA. This work was carried out with Dr. M. Murphy and Dr. P. Farr.

2. Semi-quantitative assessment of UV-irradiated skin: An observer who was unaware of treatment conditions (as all slides were coded by another observer) assessed the number of positively stained epidermal nuclei. Cells showing nuclear staining were assessed in an ascending horizontal layer in basal layer, suprabasal and high suprabasal layers (in practice it was often impossible to count more than 4 to 5 consecutive layers). Cells displaying only marginal staining indistinguishable from the background were not counted. UVR effect on NFAT-5/TonEBP localization was measured by counting the number of NFAT-5 -positive nuclei per 300 μ m length of basement membrane.

Statistical analysis

To compare the effect of CsA on the number of cells showing positive nuclear immunostaining, Chi squared analysis was used. Data were analyzed using Arcus Quickstat (biomedical version 1.0). Two-way analysis of variance (ANOVA) test was used to compare the UVA and UVB effects on the number of cells showing positive NFAT-5 nuclear staining. MINITABTM statistical software (release 13) (MINITAB Ltd, UK) was used to analyze UV

effects on NFAT-5 nuclear localization.

Results

Expression of NFAT-5 mRNA in cultured keratinocytes and cultured fibroblasts

RT-PCR of keratinocyte and fibroblast cDNA, using NFAT-5 specific primers, produced a 340bp fragment as predicted, demonstrating the presence of NFAT-5 in human epidermal keratinocytes and cultured dermal fibroblasts (**Figure 2A** and **B**). Sequencing of RT-PCR products followed by BLAST analysis confirmed the identity of the product that showed 100 % homology with the predicted NFAT-5 sequence (Accession#NM_006599). cDNA from Jurkat T cell mRNA was amplified as a positive control in this experiment.

Western analysis confirms expression of NFAT-5 in cultured human keratinocytes and dermal fibroblasts

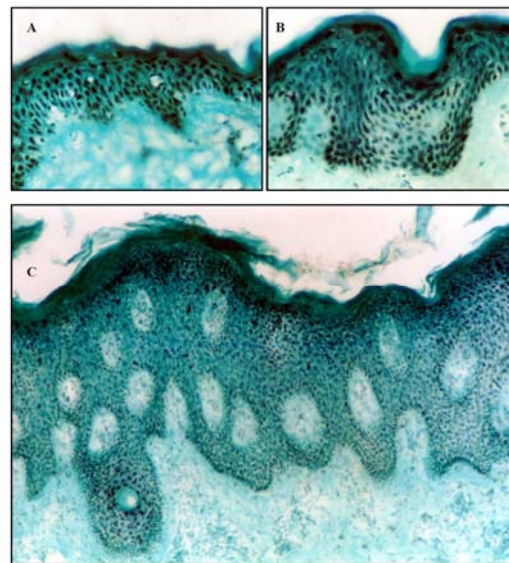


Figure 3. Immunostaining of normal skin, lesional (plaque) and non-lesional psoriatic skin (subject 1) with an anti-NFAT-5 antibody. Frozen sections of normal human skin (A) (original magnification X25), lesional (C) (plaque) (original magnification X10) psoriatic skin and non-lesional (B) (uninvolved) (original magnification X25) psoriatic skin were stained with anti-NFAT-5 antibody. NFAT-5 shows predominantly nuclear localization in normal and psoriatic skin, but there is reduced expression in suprabasal spinous layer in psoriatic skin compared to normal skin.

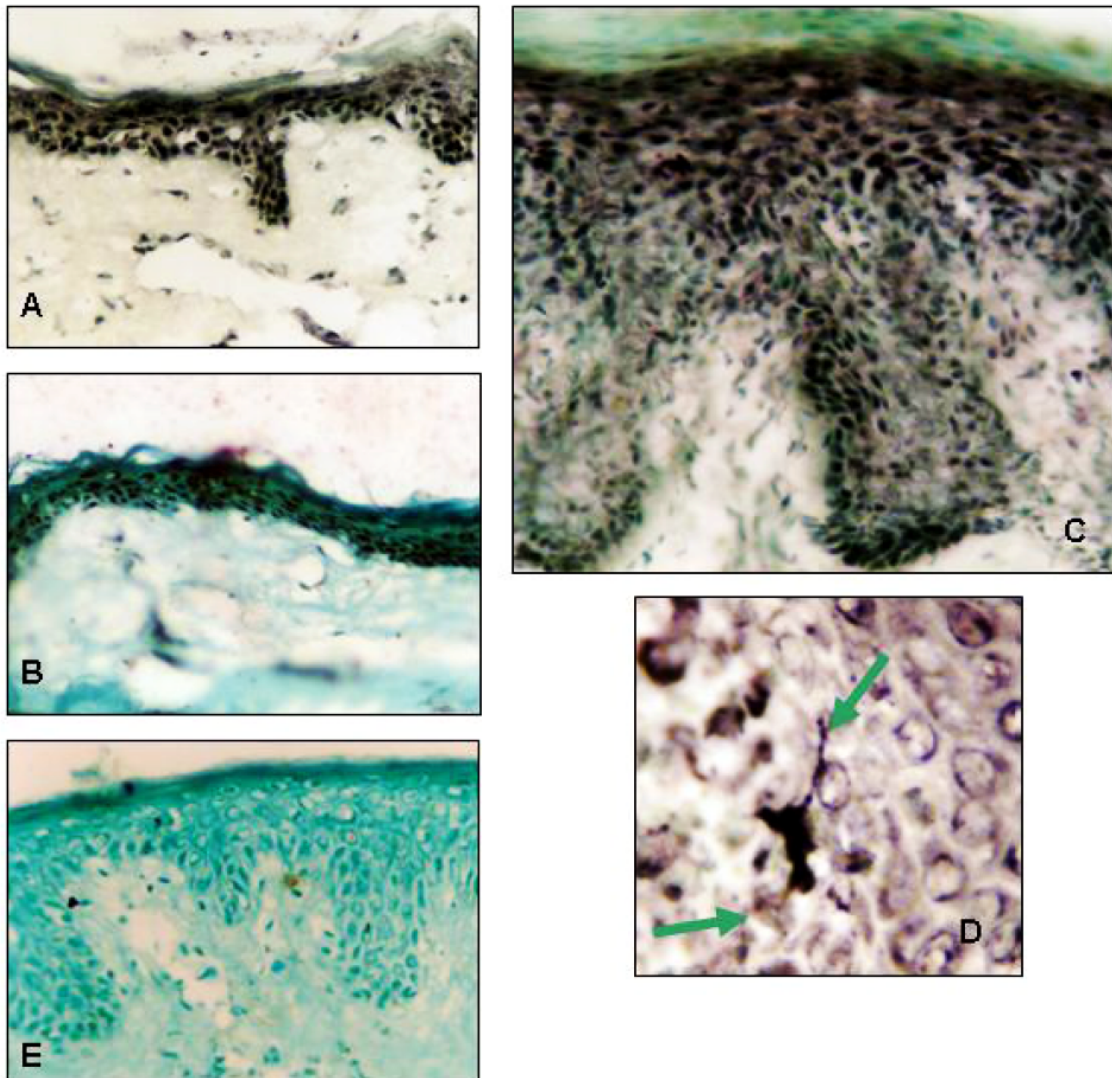


Figure 4. Immunostaining of normal skin, lesional (plaque) and non-lesional psoriatic skin (Subject 2) with an anti-NFAT-5 antibody and expression of NFAT-5/TonEBP by Langerhans cells in psoriatic skin. Frozen sections of normal human skin (A), non-lesional (uninvolved) (B) psoriatic skin and lesional (plaque) psoriatic skin (C) were stained with anti-NFAT-5 antibody. NFAT-5 shows predominantly nuclear localization in normal and psoriatic skin. Langerhans cells express NFAT-5 in the suprabasal layer of lesional psoriatic skin (D). Arrowheads indicate dendritic processes of the Langerhans cell. (E) frozen sections of normal skin stained with goat IgG as negative control. (Original magnification x25).

Western blotting showed that cultured human keratinocytes express NFAT-5/TonEBP protein. These experiments also demonstrated that the antibody used in immunohistochemistry and immunofluorescence/confocal microscopy staining detected the appropriate molecular weight protein in skin cells (202 kDa). In addition, anti-NFAT-5 mainly detected a single band in cultured keratinocytes and dermal fibroblasts (Figure 2C).

Expression of NFAT-5/TonEBP in normal and psoriatic skin

Immunohistochemistry showed that epidermal keratinocytes and dermal fibroblasts in normal human and psoriatic skin express NFAT-5 (Figure 3 and Figure 4). NFAT-5 showed predominantly nuclear localization in epidermal keratinocytes and dermal fibroblasts within five normal adult skin

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Table 1. Distribution of NFA 5/TonEBP in normal (A) and psoriatic skin (B)

<i>(A) Normal skin.</i>				
	Basal	Suprabasal	High suprabasal	
Subject 1	4 N	3 N	4 N	
Subject 2	4 N	4 N	4 N	
Subject 3	4 N	2 N/C	2 N/C	
Subject 4	4 N	4 N	4 N	
Subject 5	4 N	4 N	4 N	

<i>(B) Psoriatic skin</i>				
	Skin type	Basal	Suprabasal	High suprabasal
Subject 1	Lesional	3 N	2 N/C	4 N
	Non-lesional	4 N	2 N/C	4 N
Subject 2	Lesional	3 N	2 N	4 N
	Non-lesional	3 N	2 N/C	3 N
Subject 3	Lesional	4 N	3 N/C	4 N
	Non-lesional	4 N	2 N/C	2 N
Subject 4	Lesional	3 N	2 C	3 N
	Non-lesional	4 N	3 N	2 N
Subject 5	Lesional	4 N	2 N/C	4 N
	Non-lesional	3 N	3 N	3 N

biopsies. In normal skin, nuclear localization of NFAT-5/TonEBP was observed throughout the whole epidermis. In lesional psoriatic skin and to a lesser extent in non-lesional psoriatic skin, the intensity of the nuclear staining diminished within the lower spinous cell layers. Nuclear staining increased in high spinous and granular layers (**Table 1**). These results were observed in four of five involved psoriatic skin sections (**Table 1**). Interestingly, expression of NFAT-5/TonEBP by Langerhans cells was observed in lesional psoriatic skin (**Figure 4D**), whereas no clear immunostaining of Langerhans cells was seen in normal skin.

Optimisation of fixation method for immunofluorescence studies of NFAT-5/TonEBP and negative controls

Different fixatives did not result in any significant differences in the distribution of NFAT5 in epidermal human keratinocytes (data not shown), although fixation with methanol/acetone showed more intense staining on the cytoplasm and nuclear membrane. 0.4% paraformaldehyde then 0.2% Triton X-100 was used in subsequent experiments. Goat IgG was included at equivalent concentrations as the primary antibody in immunohistochemical (**Figure 3**) and immunofluorescence (**Figure 4A**) studies as a negative control.

Hypertonicity induces nuclear localization of the transcription factor NFAT-5/TonEBP

NFAT-5 activation by hypertonicity was investigated in cultured keratinocytes. Immunocytochemical analysis of untreated cultured keratinocytes showed that NFAT-5/TonEBP is present in both cytoplasm and nucleus. In keratinocytes cultured in hypertonic medium using 200 mM raffinose, the nuclear staining increased while the cytoplasmic staining decreased. The overall appearance of the nuclear/cytoplasmic boundary sharpened after 24 h of switching to a hypertonic medium (**Figure 5**). Redistribution occurred over a time course of 24-48 h. Absent nucleolar staining was also observed in these experiments.

Differentiation promoting agents do not cause any change in the subcellular localization of NFAT-5 in cultured human keratinocytes

Human keratinocytes cultured on coverslips in low calcium MCDB 153 medium and treated with either DMSO (vehicle control) or agonists that induce keratocyte differentiation. The addition of ionomycin (1 μ M), TPA (50 nM) or TPA (50 nM) plus ionomycin (1 μ M) for 24 h and 48 h did not result in changes in the subcellular localization of NFAT-5 in human keratinocytes (**Figure 6**).

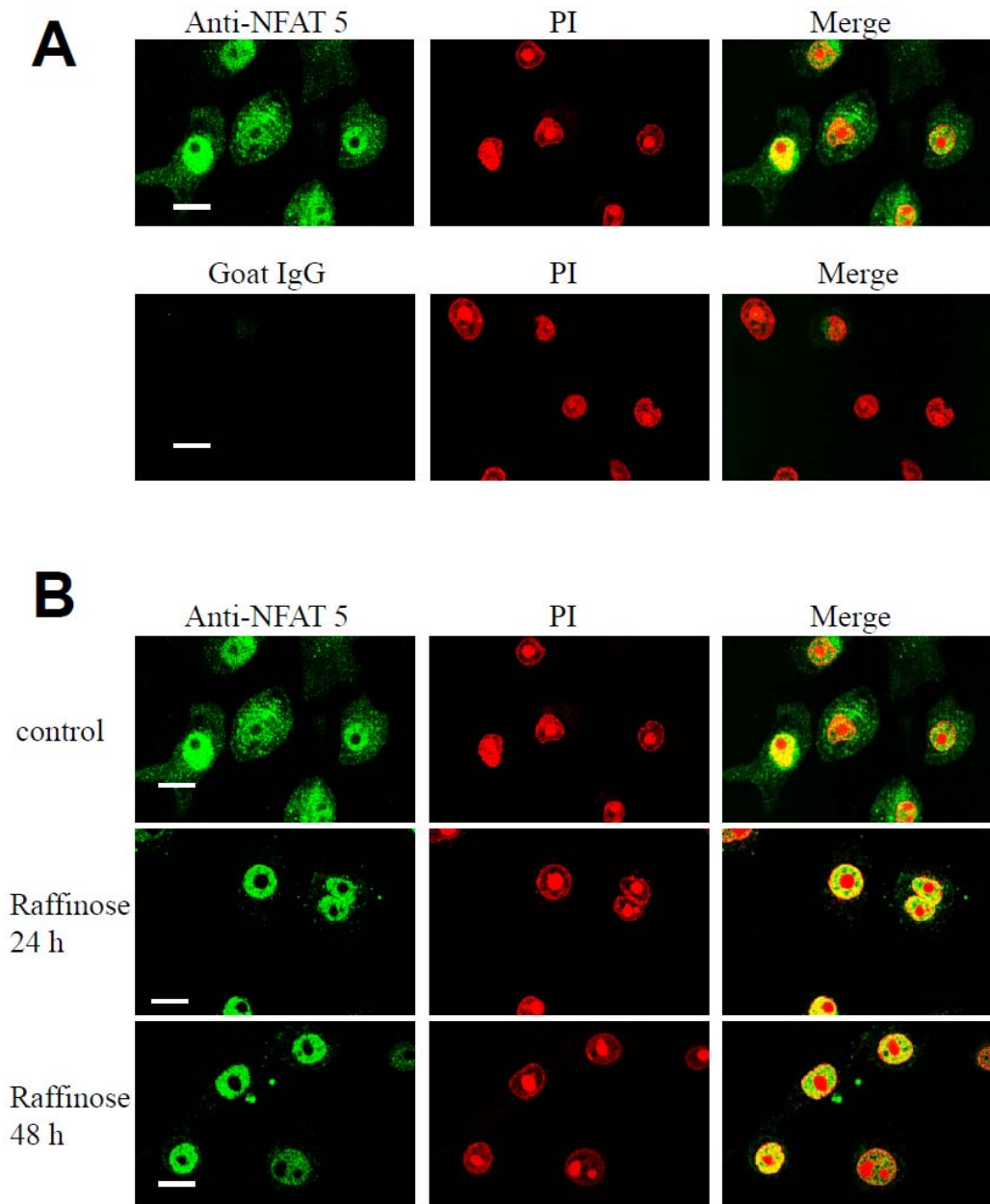


Figure 5. Anti-NFAT-5 staining specificity in cultured human keratinocytes (A). Hypertonicity induces nuclear localization of NFAT-5/TonEBP in human keratinocytes (B). (A), human keratinocytes were cultured on coverslips in low calcium MCDB 153 medium. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, incubated sequentially with goat-polyclonal anti-NFAT-5 antibody (2 $\mu\text{g}/\text{ml}$) or goat IgG (2 $\mu\text{g}/\text{ml}$), rabbit anti-goat FITC, propidium iodide (50 $\mu\text{g}/\text{ml}$) and visualized using a Biorad confocal microscope. The images shown are mid-cell sections. Negative control coverslip (goat IgG) was scanned using the same settings (gain, black level and confocal aperture) as the positive control coverslip (anti-NFAT-5), thus ensuring that the pixel brightness values were due to antibody labelling rather than other factors such as autofluorescence or non-specific binding. Pixel brightness data were analyzed using COMOS software. (B), human keratinocytes were cultured on coverslips in low calcium MCDB 153 medium (control) and then switched to medium containing 200nM of raffinose for 24 h and 48 h as indicated. Cells were fixed and incubated sequentially with goat-polyclonal anti-NFAT-5 antibody, rabbit anti-goat FITC, propidium iodide (50 $\mu\text{g}/\text{ml}$) and visualized using a Biorad confocal microscope. The images shown are mid-cell sections. These results are representative of 3 experiments on keratinocytes derived from 3 independent donors. Scale bar 25 μM .

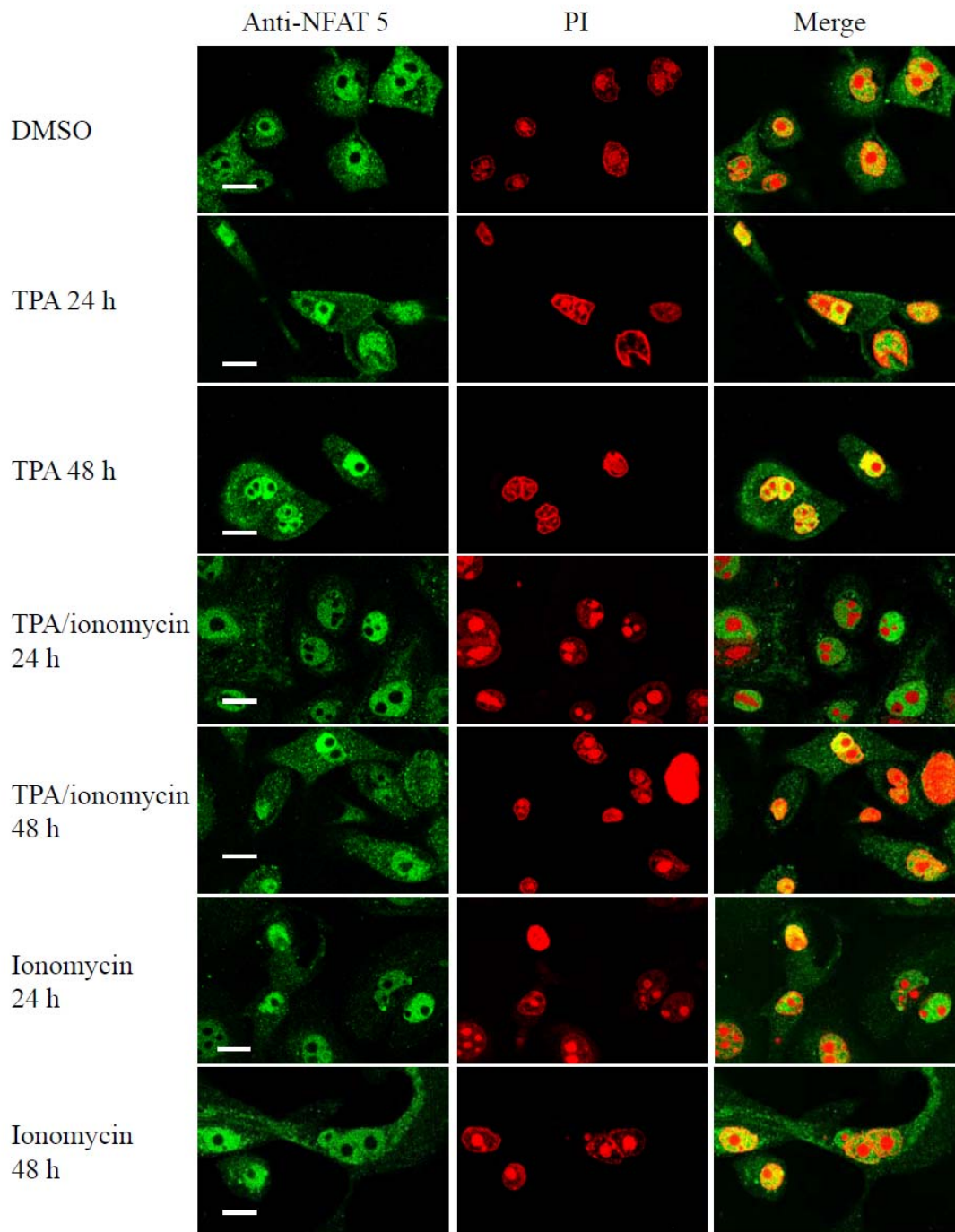


Figure 6. Differentiation promoting agents do not cause any change in the subcellular localization of NFAT-5/TonEBP in human keratinocytes . Human keratinocytes were cultured on coverslips in low calcium MCDB 153 medium (control) and then then treated with DMSO (vehicle control), ionomycin (1 μ M), TPA (50 nM) plus ionomycin (1 μ M) or TPA (50 nM) for 24 h and 48 h as indicated. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, incubated sequentially with goat-polyclonal anti-NFAT-5 antibody, rabbit anti-goat FITC, propidium iodide (50 μ g/ml) and visualized using a Biorad confocal microscope. The images shown are mid-cell sections. These results are representative of 3 experiments on keratinocytes derived from 3 independent donors. Scale bar 25 μ M.

Table 2. Alteration of NFAT5 nuclear localization in human keratinocytes

Treatment	% cells showing NFAT5 nuclear positivity	Number of cells counted
Medium control*	100	150
DMSO control†	100	150
Raffinose 24h	100	150
Raffinose 48h	100	150
TPA/ionomycin‡ 24h	100	150
TPA/ionomycin‡ 48h	100	150
Ionomycin 24h	100	150
Ionomycin 48h	100	150
TPA 50 nM 24h	100	150
TPA 50 nM 48h	100	150
CsA 1 µM 24h	100	150
CsA 1 µM 48h	17.3§	150
Tacrolimus 1 µM 24h	100	150
Tacrolimus 1 µM 48h	100	150

* Low calcium medium; †Vehicle control; ‡TPA (50 nM) plus ionomycin (1 µM), §P<0.0001 compared to DMSO control.

Cyclosporin A induces nuclear membrane translocation of NFAT-5/TonEBP in cultured keratinocytes

There is controversy regarding the role of calcineurin in regulating NFAT-5/TonEBP in T cells and kidney cell line [2, 5, 6, 47]. CsA and tacrolimus have been shown to inhibit activation of both p38 and JNK pathways in T cells in a calcineurin-independent way, suggesting that p38/JNK signaling pathways are activated in a CsA sensitive manner and contribute to IL-2 gene expression in T lymphocytes [28, 29]. Therefore, CsA and tacrolimus effects on NFAT-5/TonEBP might be mediated via either JNK/p38 pathways or the calcineurin pathways. In untreated cultured keratinocytes or cells treated with a vehicle, NFAT-5 was found in both the nucleus and the cytoplasm (100% nuclear positivity). Treatment of cultured keratinocytes with CsA (1 µM) for 48 h significantly induced nuclear membrane translocation of NFAT-5/TonEBP and resulted in a decrease in the number of cells showing nuclear positivity (17.3% nuclear positivity, P<0.0001). Furthermore, a decrease in the cytoplasmic staining was also observed. These results were observed in three independent experiments (**Figure 7** and **Table 2**).

Tacrolimus (FK506) does not cause significant changes in the subcellular localization of NFAT-5/TonEBP in cultured keratinocytes

Human keratinocytes were treated with tacrolimus (1 µM) for 24 and 48 h, then immunostained with antibody against NFAT5/TonEBP. Tacrolimus did not result in significant changes in the nuclear localization of NFAT-5/TonEBP in cultured keratinocytes (**Figure 7** and **Table 2**). However, a decrease in the cytoplasmic staining was observed.

Effects of UVA and UVB on NFAT-5/TonEBP localization in human epidermis in vivo

Skin biopsies were taken from areas of approximately equal (moderate) erythema following irradiation with UVA and UVB. Biopsies were also taken from un-irradiated (control) skin. The mean (±SD) irradiation doses at the biopsy sites were 44 ± 8 mJ/cm² and 37 ± 6 J/cm² for UVB and UVA respectively. The mean (±SD) erythema readings from the biopsy sites were 223 ± 34 and 208.7 ± 28 (arbitrary units) for UVB and UVA irradiation sites respectively. NFAT5/TonEBP protein expression was assessed by immunocytochemistry. NFAT-5 staining was mainly nuclear throughout the epidermis (**Figure 8**). The mean number of cells showing nuclear positivity in both UVA and UVB-irradiated skin was reduced compared to un-irradiated skin (**Table 3** and **Figure 8**). Two-way ANOVA was used to make individual comparisons between different factors that may affect the results (e.g. subjects and UV

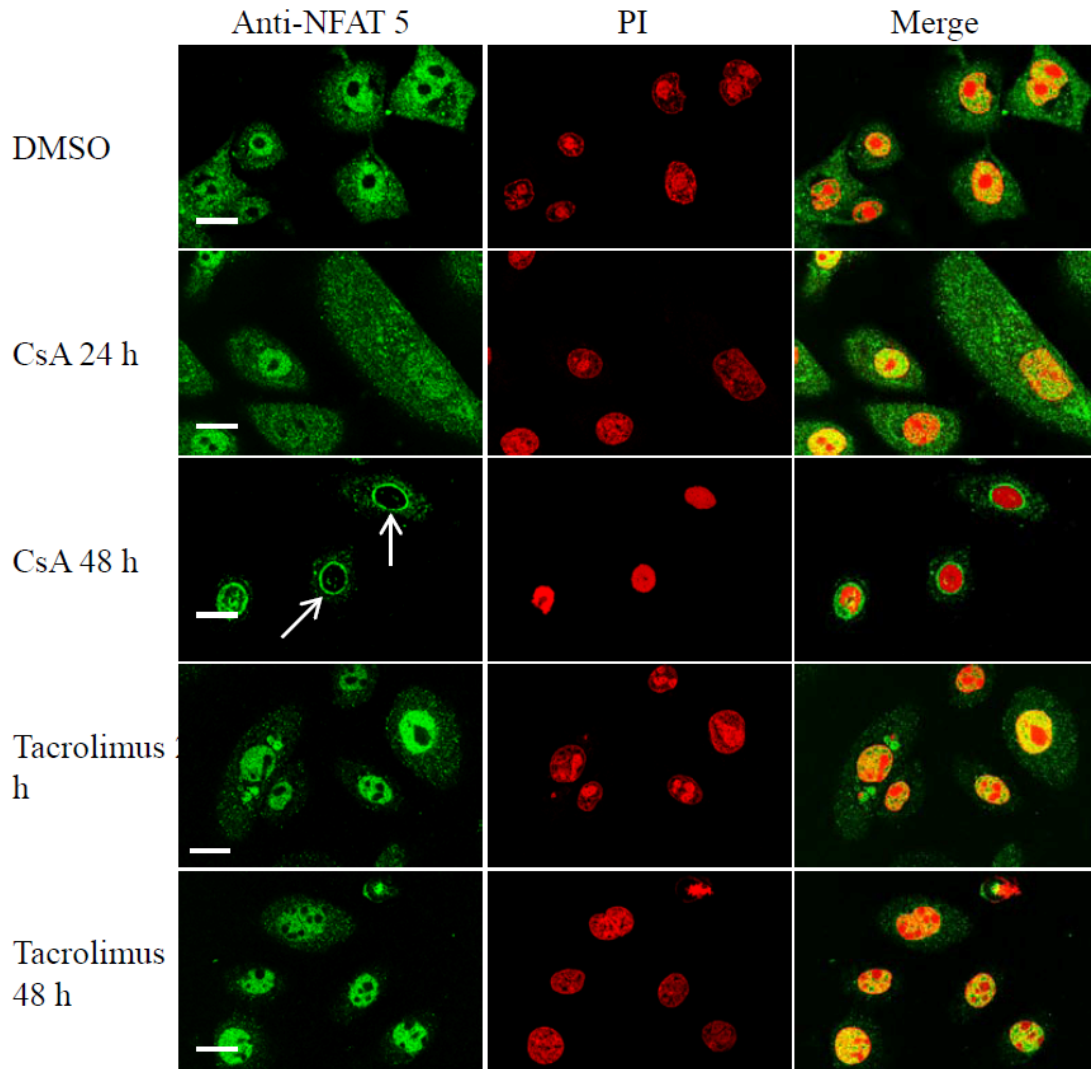


Figure 7. Cyclosporin A, but not tacrolimus, induces nuclear membrane translocation of NFAT-5/TonEBP in human keratinocytes. Human keratinocytes were cultured on coverslips in low calcium MCDB 153 medium (control) and then then treated with DMSO (vehicle control), CsA (1 μ M) or tacrolimus (1 μ M) for 24 h and 48 h as indicated. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, incubated sequentially with goat-polyclonal anti-NFAT-5 antibody, rabbit anti-goat FITC, propidium iodide (50 μ g/ml) and visualized using a Biorad confocal microscope. The images shown are mid-cell sections. These results are representative of 3 experiments on keratinocytes derived from 3 independent donors. Arrowheads indicate nuclear membrane translocation of NFAT-5/TonEBP in response to CsA. Scale bar 25 μ M.

irradiation). The variance between subjects was not significant ($P=0.46$). When UVA and UVB were combined, the variance in the numbers of cells showing NFAT-5 positivity between UV-irradiated and un-irradiated skin approached significance ($P=0.06$). Further analysis indicated that there were no significant differences in the variance of NFAT-5 nuclear positive cells between UVA and UVB irradiated epidermis. These data suggest that

UV irradiation (UVA and UVB) reduces NFAT-5 nuclear localization within the epidermis.

Discussion

Although a northern blot analysis detected NFAT-5/TonEBP transcripts in many human tissues, normal human skin was not included in that study [5]. Therefore, our studies provide the first observation of expression of NFAT-

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Table 3. Summary of NFAT-5 cells showing nuclear positive cells in normal (un-irradiated), UVB-irradiated and UVA irradiated epidermis. The number of NFAT-5-positive nuclei per 300 μm length of basement membrane is shown here.

	Normal (un-irradiated skin)	UVB-irradiated skin	UVA-irradiated skin
Subject 1	110	34	7
Subject 2	101	51	26
Subject 3	105	47	108
Subject 4	45	63	29
Total	361	195	170
Mean	90.3	48.8	42.5
Standard Deviation	30.4	12	44.7

5/TonEBP mRNA protein in cultured keratinocytes and dermal fibroblasts and possible functional regulation in cultured keratinocytes.

Changes in humidity have profound effects on skin barrier function by creating an osmotic gradient across the stratum corneum [48]. These effects are more severe when the barrier function of the stratum corneum is disrupted in skin disorders such as psoriasis [48]. Our study has also shown that normal human skin and psoriatic skin express the transcription factor NFAT-5/TonEBP that regulates tonicity-responsive expression of proteins that catalyze cellular accumulation of compatible osmolytes. By immunostaining of normal skin and uninvolved psoriatic skin, NFAT-5/TonEBP appeared predominantly nuclear in all skin layers. However, NFAT-5/TonEBP in involved psoriatic skin appeared to predominantly localize to both the basal and high suprabasal but not the low suprabasal layers. The interpretation of this data is at present unclear and requires further study.

It is well known that CsA and tacrolimus through formation of a complex with cyclophilin and FKBP12, inhibit the phosphatase activity of calcineurin, which regulates nuclear translocation and subsequent activation of NFAT transcription factors. In addition to the calcineurin/NFAT pathway, recent studies indicated that CsA and tacrolimus also block the activation of JNK and p38 signaling pathways triggered by antigen recognition [26, 28, 29]. As NFAT 4 in dermal fibroblasts, nuclear membrane translocation of NFAT-5/TonEBP in response to CsA (1 μM) provide extra evidence of CsA direct effects on keratinocytes, which may account in part for

the therapeutic effect of CsA in skin diseases such as psoriasis. It would be interesting to know whether CsA induced-nuclear membrane translocation of NFAT-5/TonEBP can be blocked by pre-treating cells with another calcineurin inhibitor such as tacrolimus or inhibitors of JNK/p38 pathways such as SP600125 (JNK inhibitor) [49] or SB-203580 (p38 inhibitor) [47].

Although the main NFAT activation pathways are distinct, there is evidence that cross-talk may occur. MAP kinases are involved in regulating NFAT activation. The MAP kinase p38 has been reported to phosphorylate in vitro and interacts in vivo with NFAT 1 to prevent its nuclear translocation [50], and p38 has been suggested to phosphorylate and inhibit NFAT 2 activation [51]. Also JNK was reported to phosphorylate NFAT 4 and control its cellular localization [52]. Later, another study contradicted these findings by showing that JNK does not inhibit NFAT 4 nuclear import [53]. Recently, JNK1 was shown to phosphorylate specific residues in the PxIxIT calcineurin-targeting motif of NFAT 2, thus inhibiting the interaction between NFAT 2 and calcineurin, although the corresponding region of NFAT 1 is not phosphorylated in stimulated cells [54]. In addition, T cells from JNK1^{-/-} mice showed increased nuclear localization of NFAT 2 but not NFAT 1, suggesting that JNK may suppress the activation of NFAT 2 [55]. Furthermore, the calcium requirement for JNK activation in T and B cells appears to be mediated by calcineurin, as it is blocked by CsA [27, 56]. However, tacrolimus effects on NFAT-5/TonEBP localization were different from that of CsA in cultured keratinocytes. This cross talk between different pathways may

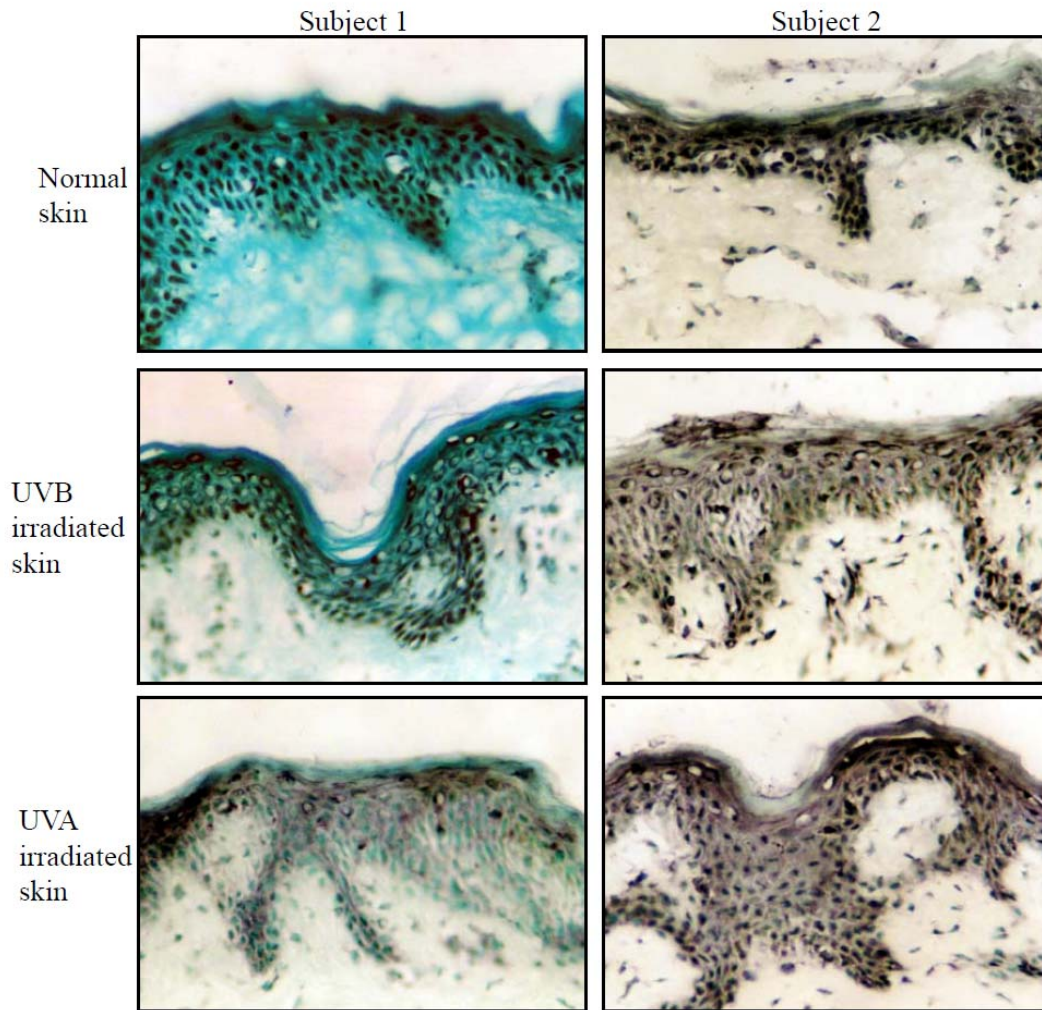


Figure 8. Effects of UVA and UVB on NFAT-5/TonEBP localization in human epidermis *in vivo*. 4 mm punch biopsies were taken from un-irradiated, UVA and UVB-irradiated sites of equal erythema as described in section 5.2.2. Sections were stained with goat polyclonal anti-NFAT-5 and the number of positively stained epidermal nuclei was counted as described. (Original magnification x25).

help to explain the effects of CsA and tacrolimus on NFAT-5/TonEBP activation in human epidermal keratinocytes. Although both CsA and tacrolimus clear psoriasis, it is also likely that there are distinct targets for CsA and tacrolimus in skin. For example, although topical tacrolimus is effective in atopic eczema [57], topical tacrolimus is only partially effective in psoriasis when applied under occlusion [58] even though the barrier in psoriasis is known to be disrupted. However, the barrier is not as disrupted in psoriasis as in eczema.

Shore reported clearing of psoriatic lesions

following prolonged occlusion with application of tapes [59, 60]. Friedman showed in a prospective bilateral comparison study that hydrocolloid occlusion had a similar effect on small psoriatic lesions [61]. Another clinical and immunohistologic study has shown that prolonged occlusion is an effective treatment for psoriasis either as monotherapy or in combination with a high potency topical steroid [62]. The mechanism of action of prolonged occlusion is still unknown. Occlusion therapy comprises the delivery of mechanical pressure to the epidermis. Different mechanical stimuli lead to membrane deformation in skin cells [63, 64].

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Hyperosmotic stress leads to cell shrinkage, causing a fast increase in the ratio of cell surface to volume. The cellular deflation over a rigid skeleton leads to both membrane stretching and folding of the plasma membrane in various areas, thus mimicking the mechanical stress. Hence, a mechanical load applied to cells can be mimicked by hypertonic stimuli [65]. Furthermore, another group demonstrated that osmotic stress causes structural changes at the level of macromolecules via crowding and hydration [66]. They replaced, for example, a hydrostatic pressure with osmotic stress based on maintaining a concentration gradient between a concentrated bathing solution and a water-filled channel leading to a decrease in the channel volume. Recently, a hyperosmotic stimulus was shown to result in elevation of intracellular calcium and inhibition of the proliferation of HaCaT keratinocyte cell line [67]. Studies in this area have shown that switching keratinocytes to a hypertonic medium resulted in localization of NFAT-5/TonEBP to the nucleus, suggesting activation of this transcription factor in response to osmotic stress. Very recently, NFAT-5/TonEBP redistribution into the nucleus of dermal fibroblasts isolated from normal human foreskin in response to hypertonic stimulus after 90 min has been reported [68]. Thus, NFAT-5/TonEBP may be involved in mediating the action of occlusive therapy in psoriasis. It would also be interesting to investigate whether calcineurin inhibitors can inhibit the hypertonicity-induced NFAT-5/TonEBP nuclear localization. However, hyperosmolarity, the main NFAT-5/TonEBP stimulus, is not blocked by the inhibition of calcineurin in T cells [5].

Unlike NFAT 1-4, NFAT-5/TonEBP was localized to both nucleus and cytoplasm of cultured keratinocytes. In addition, differentiation-promoting agonists that induce sustained rise in intracellular calcium did not result in changes in NFAT-5/TonEBP localization in cultured keratinocytes, suggesting that the effects of rapamycin and CsA are not mediated by an increase in the intracellular calcium concentrations. These distinct features of NFAT-5/TonEBP in human cultured keratinocytes support the hypothesis that NFAT-5/TonEBP is an outlying member of the NFAT family [16].

Keratinocytes are exposed to the carcinogenic effects of UVR. UV-induced non-melanoma

skin cancer is thought to arise from damaged basal keratinocytes and p53, a tumor suppressor, is a potential candidate involved in protecting basal keratinocytes from UVR effects [69, 70]. Using indirect immunofluorescence/confocal microscopy, UV radiation was shown to induce nuclear translocation of NFAT 2 in an epidermal cell line [71]. Investigation of UV effects on NFAT-5/TonEBP nuclear localization in vivo suggested that UVR (UVA and UVB) reduce nuclear localization in human epidermis ($P=0.06$ compared to unirradiated skin, $n=4$). However, further studies including more irradiated biopsies may prove useful. These results provide the first description of NFAT-5/TonEBP export from the nucleus. However, this nuclear export mechanism is unclear at the moment.

In summary, the CsA and rapamycin effects on NFAT-5/TonEBP in cultured keratinocytes suggest diverse intracellular signaling pathways for NFAT-5/TonEBP in these cells, and that NFAT-5/TonEBP might function to translate different extracellular stimuli into appropriate functional responses. Also CsA and rapamycin have a T cell-independent effect on epidermal keratinocytes and may have two distinct mechanisms of action. As in T cells, one is the inhibition of calcineurin phosphatase activity and the other is the calcineurin-independent inhibition of JNK/p38 activation pathways [28, 29].

We have previously shown that treatment of cultured human keratinocytes with agents that induce a sustained rise in intracellular calcium, including elevation of extracellular calcium ($[Ca^{2+}]_o$) leads to nuclear translocation of endogenous NFAT1, which was inhibited by pre-treatment with CsA, rapamycin [39, 72] and recently with nifedipine [40]. These data provide the first evidence that NFAT-5 is functionally active in human keratinocytes. In contrast to other NFATs in the skin, NFAT-5 appears to be primarily located in the nucleus. These data provide the first evidence that NFAT-5 is functionally active and that CsA has a direct effect on NFAT-5 subcellular localization. Ultimately, the identification of NFAT-regulated genes in skin cells will be crucial for developing an understanding of the physiological role that these transcription factors play in skin differentiation. However, the functional significance of each NFAT member in skin remains to be fully explored.

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