

Tacrolimus does not alter the production of several cytokines and antimicrobial peptide in *Malassezia furfur*-infected-keratinocytes

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Summary

Topical immunosuppressant therapy is widely used in the treatment of inflammatory skin diseases, such as atopic dermatitis and psoriasis. Besides its beneficial therapeutic effects, application of topical anti-inflammatory drugs may render the epidermis more vulnerable to invading pathogens by suppressing innate immune responses in keratinocytes (KCs). Cytokines, chemokines and antimicrobial peptides (AMPs) produced by epithelial cells enable them to participate in innate and acquired immune responses. The aim of the present work was to study the influence of tacrolimus (FK506) on KCs infected with *Malassezia furfur* (*M. furfur*), evaluating the expression of pro-inflammatory cytokines IL-1 α and IL-6, chemokine IL-8, anti-inflammatory cytokines transforming growth factor beta1 (TGF- β 1) and IL-10 and AMP β -defensin-2. Human KCs were obtained from surgical specimens of normal adult skin. The expression of mRNAs in KCs: FK506-treated, FK506-treated and *M. furfur*-infected as well as only *M. furfur*-infected was quantified by real-time quantitative polymerase chain reaction. Next, the production of the AMP β -defensin-2 and of the above-mentioned pro-inflammatory and anti-inflammatory cytokines was evaluated using enzyme-linked immunosorbent assay. In this study, FK506 did not alter cytokine and AMP production by KCs; this led us to hypothesise that it may not enhance the risk of mycotic skin infections.

Key words: FK506, human beta-defensin-2, *Malassezia furfur*, mycotic infections.

Introduction

Tacrolimus (FK506), a macrolide isolated from *Streptomyces tsukubaensis*,¹ exerts potent immunosuppressive effects in a variety of *in vitro* and *in vivo* systems;^{2–4} in fact, it is used to prevent graft rejection after organ transplantation in animals and human beings.^{5–8} Regarding cutaneous diseases, FK506 ointment has been used successfully in chronic inflammatory

dermatoses, such as atopic dermatitis and psoriasis.^{9–12} FK506 inhibits the calcium-dependent phosphatase calcineurin, an enzyme required for the dephosphorylation of activated T cells nuclear factor cytosolic form. Therefore, the therapeutic efficacy of FK506 has been attributed to its impact on T-cell activation, making it effective for the treatment of inflammatory skin disorders.^{13,14}

Although FK506 ointment improves skin manifestations in both psoriasis and atopic dermatitis, little has been done to investigate the possible interactions between FK506 and keratinocytes (KCs).

Cytokines, chemokines and antimicrobial peptides (AMPs) produced by epithelial cells enable them to participate in innate and acquired immune responses.^{15–19} Among AMPs, β -defensins, originally isolated from lesional psoriatic skin, constitute an

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important link between innate and acquired immunity.²⁰ Human beta-defensin-2 (HBD-2) exhibits a broad spectrum of antimicrobial activity and its capacity to kill bacteria *in vivo* has been demonstrated.²¹ Expression of HBD-2 in epithelial cells is induced by pro-inflammatory cytokines as well as bacteria.²² In addition to microbicidal functions, HBD-2 induces chemotaxis of immature dendritic cells and memory T cells.²³ *Malassezia* species are a component of the cutaneous microflora that colonises lipid-rich areas, especially the head and neck, because these microorganisms require lipids for their growth. *Malassezia furfur* is not only a commensal, because it may also cause or be a co-causal factor of *pityriasis versicolor*, seborrheic dermatitis, *M. folliculitis*. Moreover, it may exacerbate both atopic dermatitis and psoriasis, two of the most common chronic inflammatory skin diseases.²⁴ *M. furfur* may overcome the skin barrier by penetrating KCs, via endocytotic process, inhibiting transglutaminase I.²⁵ Moreover, the inhibition of pro-inflammatory cytokines could allow the yeast to survive in the host cell without causing any inflammatory response. In a previous study, we demonstrated that uptake of *M. furfur* by human KCs induced the production of IL-10, transforming growth factor beta1 (TGF- β 1) and HBD-2.²⁵ HBD-2, induced by internalised *M. furfur*, limits the uptake of further *Malassezia* cells from the extracellular environment. This is particularly important in the case of *Malassezia*, which, through the induction of immunosuppressive cytokines, succeeds in surviving in human KCs. Moreover, we showed that in psoriatic patients *M. furfur* induced the upregulation of the AMP HBD-2.²⁵ The aim of the present work was to study the effects of FK506 on KCs' mycotic infection, evaluating pro-inflammatory cytokines (IL-1 α and IL-6), chemokine IL-8, anti-inflammatory cytokines (TGF- β 1 and IL-10) and AMP HBD-2 in KCs infected with *M. furfur*.

Materials and methods

Microorganism and culture media

Malassezia furfur ATCC 12078 was obtained from the American Type Culture Collection (Rockville, MD, USA). To confirm the identity of the ATCC strain we have characterised it by nested PCR of the intergenic transcribed spacer (ITS) ITS1 and ITS2 region ribosomal gene clusters, as previously reported,²⁶ proving that it effectively was *M. furfur*. It was grown for 4 days at 30 °C in modified Leeming and Notman agar composed of (per litre) 10 g glucose, 10 peptone,

8 g bile salts, 2 g yeast extract, 0.5 g glycerol monostearate, 15 g agar, 10 ml glycerol, 5 ml Tween 60 and 20 ml olive oil.

Culture and treatments of KCs

Human KCs were obtained from surgical specimens of normal adult skin (after obtaining informed consent). The skin was split overnight in dispase (Sigma-Aldrich, St. Louis, MO, USA) at 4 °C. Epidermal sheets were removed from the dermis and single-cell suspensions were obtained by placing the epidermal sheets in 0.05% trypsin/0.53 mmol l⁻¹ EDTA for 15 min at 37 °C. Following incubation, trypsin activity was stopped by adding 10 ml soybean trypsin inhibitor (Life Technologies, Carlsbad, CA, USA) and the KC cell suspensions centrifuged. The resulting cell pellet was cultured in defined KC medium (serum-free KC medium, SFM; Life Technologies) at a density of approximately 3×10^6 cells per flask in 15 ml complete medium. Cell numbers were determined in a haemocytometer chamber, using trypan blue exclusion to monitor viable cells. The total calcium concentration in the medium was maintained at 0.09 mmol l⁻¹. To confirm that KCs were undifferentiated, a transglutaminase assay was performed, as previously described.²⁷ Cultured KCs (approximately 80% confluency) were infected with *M. furfur* at a yeast cell to KCs ratio of 30 : 1 and, simultaneously, incubated with FK506 at the indicated concentrations (0, 10, 100 and 1000 nmol l⁻¹). This range was chosen on the basis of previous published papers on FK506 use in primary KCs.^{16,28,29} To execute experiments with a concentration that was not too far from the one used in clinical practice, an analysis of the literature was performed and a similar proportion was maintained among topical product concentration and concentration used in cell cultures.³⁰ Moreover, the range selected was to exclude any antifungal activities by FK506, as assessed by Sugita *et al.* [31] As we have previously assessed that after 48 h of co-culture the yeast penetrates the cytoplasm of KCs with an uptake of 30%,²⁵ this was selected as the minimum time point for the incubation with FK506. FK506 was purchased from Calbiochem-Novabiochem Corp. (San Diego, CA, USA) and dissolved in dimethylsulphoxide (DMSO) at 1 mmol l⁻¹ as stock solution. KCs were treated with FK506 at doses ranging from 10 to 1000 nmol l⁻¹. DMSO solutions from 0.001% to 1% were prepared and tested as solvent controls for KC viability, but 0.1% was then selected as the concentration for dissolving FK506. Cells were incubated with

M. furfur and/or FK506 at indicated concentrations for 48 h for RNA extraction, whereas a 72 h-time was chosen for protein analysis.

KC viability assay

The viability of KCs was assessed by 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt cell proliferation assay following manufacturer's instructions (Cell-Titer 96 aqueous proliferation assay kit; Promega, Madison, WI, USA). Viability was determined by a microplate reader with colorimetric absorbance set at 490 nm (Model 680 Microplate Reader; Bio-Rad Laboratories, Hercules, CA, USA).

Quantification of mRNA expression IL-1 α , IL-6, IL-8, IL-10, TGF- β 1 and HBD-2

The expression of mRNAs in KCs: FK506-treated, FK506-treated and *M. furfur*-infected and only *M. furfur*-infected were quantified by real-time quantitative polymerase chain reaction (PCR). Total RNA was purified using a High Pure RNA isolation kit (Roche Diagnostics, Mannheim, Germany). cDNA was obtained using an expand reverse transcriptase (Roche Diagnostics) according to the manufacturer's instructions and subjected to real-time PCR analysis (LightCycler; Roche Diagnostics). Each real-time PCR was done three times for each sample. After completion of the amplification protocol, a melting curve analysis was performed to confirm the specificity of the amplified product. Fluorescence emission readings were monitored and analysed by LightCycler Data Software (Roche Diagnostics). The specificity of the amplification products was verified by electrophoresis on a 1.4% agarose gel stained with ethidium bromide. Sequence-specific standard curves were generated using serial dilution of specific DNA standards. The data are presented as the ratio between the target gene expression, normalised to the housekeeping gene β -actin, vs. unstimulated conditions.

Measurements of IL-1 α , IL-6, IL-8, IL-10, TGF- β 1 and HBD-2 in KC supernatants using an enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-1 α , IL-6, IL-8, IL-10, TGF- β 1 and HBD-2 in the cell-free supernatants derived from KCs, were measured 72 h after treatments by commercially available IL-1 α , IL-6, IL-8, IL-10, TGF- β 1 ELISA kits according to the manufacturer's instructions

(Quantikine; R&D Systems, Minneapolis, MN, USA). The limits of quantification of this assay were 1.6–1000 pg ml⁻¹. Analysis of HBD-2 was performed by an ELISA kit according to the manufacturer's instructions (Phoenix Pharmaceuticals, St Joseph, MO, USA), which had a detection limit of 2.3 pg ml⁻¹.

Statistical analysis

Each experiment was performed at least five times. The results are expressed as means $\pm$ standard deviations. The ANOVA test (analysis of variance between groups) was performed for each kind of experiment. $P < 0.05$ was considered significant.

Results

FK506 treatment did not significantly affect KC viability

FK506 did not significantly influence human KCs morphology, at any dose tested (data not shown). The treatment of human KCs with FK506 at the 10, 100, 1000 nmol l⁻¹ and, simultaneously, infected with *M. furfur* did not significantly alter the cell viability, as seen in Fig. 1. No difference in viability was appreciated regardless of DMSO concentration (data not shown).

Effects of FK506 on cytokine expression in human KCs infected with *M. furfur*

Keratinocytes have been reported to participate in the innate defence by expressing immune effectors such as

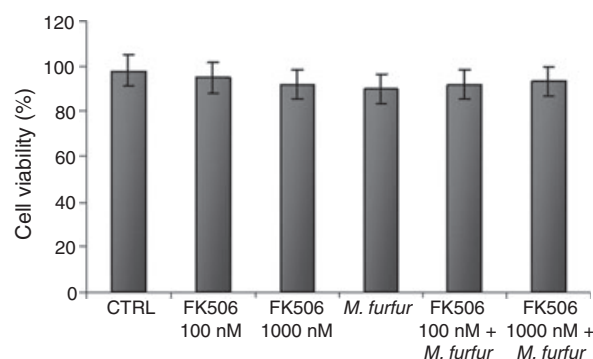


Figure 1 Keratinocytes (KCs) viability at 48 h after incubation with: FK506 at different concentrations (100 and 1000 nmol l⁻¹), *Malassezia furfur* only (30 : 1) as well as simultaneous treatment with *M. furfur* (30 : 1) and FK506 (100 and 1000 nmol l⁻¹). Data are displayed as mean $\pm$ standard deviation of five independent experiments, each performed in duplicate; CTRL (control): unstimulated healthy KCs.

cytokines and chemokines in response to microorganisms. To determine the effects of FK506, KCs were cultured in the presence/absence of *M. furfur* and treated with 100 nmol l⁻¹ or 1000 nmol l⁻¹ FK506. After 48 h, total RNA was extracted and analysed by real-time quantitative PCR for the expression of IL-1 α , IL-6, IL-8, IL-10 and TGF- β 1. As shown, in Fig. 2a, after treating KCs with 100 nmol l⁻¹ or 1000 nmol l⁻¹ FK506 together with *M. furfur*, the increase in TGF- β 1 and IL-10 mRNA was significantly higher than that in KCs control. The addition of FK506 did not significantly alter the effect of *M. furfur* on TGF- β 1 and IL-10 mRNA expression in KCs. The ELISA assay confirmed data about IL-10, whereas for TGF- β 1 the increase obtained after concomitant treatment with FK506 (100 and 1000 nmol l⁻¹) and *M. furfur* was

higher respect to the infection with the yeast only (Fig. 2b). Neither FK506 nor *M. furfur* affected IL-1 α and IL-6 expression, whereas IL-8 expression was induced by *M. furfur* but not altered by FK506 (Fig. 3a). The ELISA assay (Fig. 3b) confirmed the data obtained by real-time PCR.

Effects of FK506 on HBD-2 expression in human KCs infected with *M. furfur*

The production of HBD-2 by KCs represents one of the primary innate immune response in the host against microorganism such as *M. furfur*. To study the expression of HBD-2, human KCs infected or not infected with *M. furfur* were incubated in presence and absence of 100 nmol l⁻¹ or 1000 nmol l⁻¹ FK506. FK506 did

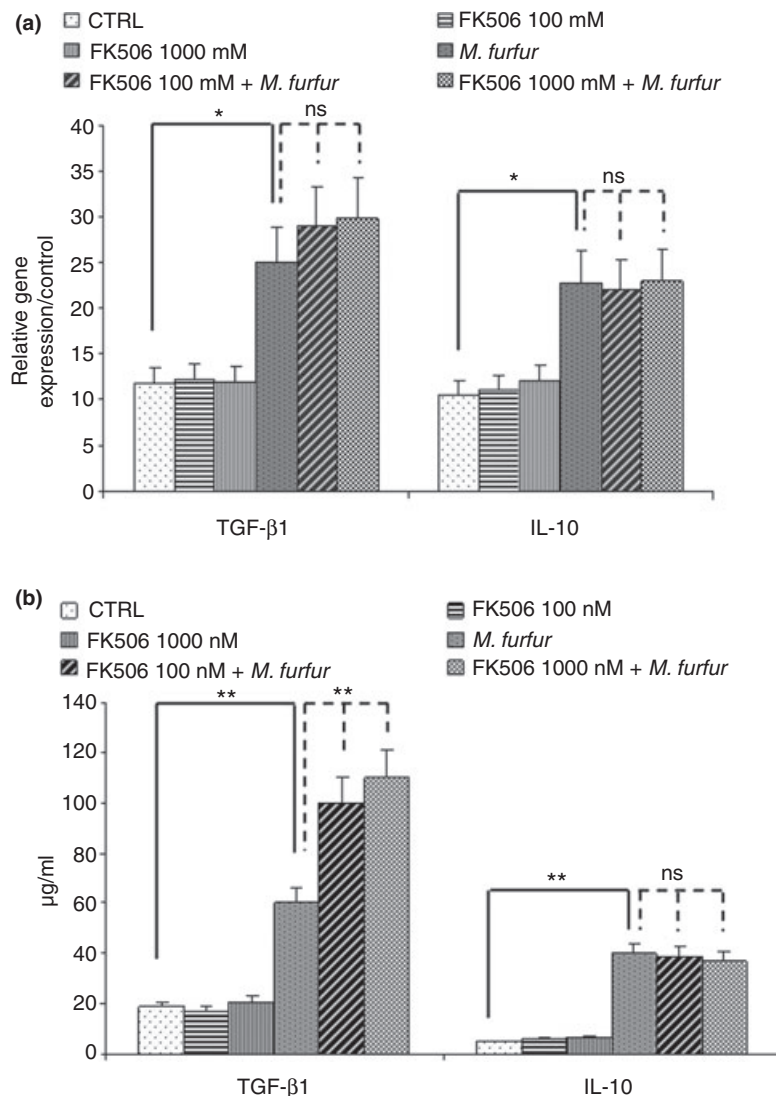


Figure 2 Effects of FK506 on transforming growth factor (TGF)- β 1 and IL-10 in human keratinocytes (KCs) infected with *Malassezia furfur*. (a) TGF- β 1 and IL-10 gene expression in KCs at 48 h after treatments; values are expressed as mRNA relative to the housekeeping gene β -actin. (b) ELISA assay. TGF- β 1 and IL-10 release from KCs at 72 h after treatments. Data are displayed as mean $\pm$ standard deviation of five independent experiments, each performed in duplicate. The ANOVA test (analysis of variance between groups) was performed for each kind of experiment. ns, Not significant; * P < 0.05; ** P < 0.01.

not modify HBD-2 gene expression induced by internalised *M. furfur* (Fig. 4a). The ELISA assay confirmed data obtained by real-time PCR (Fig. 4b).

Discussion

The aim to investigate the effects of FK506 on KCs infected with *M. furfur* had been inspired by our previous studies demonstrating that this specific infection was able to enhance IL-10, TGF- β 1 and HBD-2 gene expression.^{25,32,33} First of all, we analysed KCs viability comparing: (i) FK506 at different concentrations (100, 1000 nmol l⁻¹), (ii) *M. furfur* and (iii) simultaneous treatment with *M. furfur* and FK506. No differences were detected; this finding enabled us to rely on

similar cell viability and no toxic effects after various stimuli. We found that *M. furfur* enhanced TGF- β 1 production by KCs and that FK506 was able to potentiate this increment in a dose-dependently way. Our group has, previously, demonstrated this effect on TGF- β 1 by *M. furfur* suggesting that it can induce the overproduction of molecules involved in cell migration and hyperproliferation.³³ Epithelial cells are exquisitely sensitive to growth inhibition by TGF- β 1, in fact its role as anti-proliferative factor in this cell type is notorious.³⁴ TGF- β family members are important mediators of cell-cell and cell-environmental sensing in the dynamic processes that are responsible for tissue development and homeostasis in many organ systems.³⁴ The ability of FK506 to potentiate *M. furfur*-induced

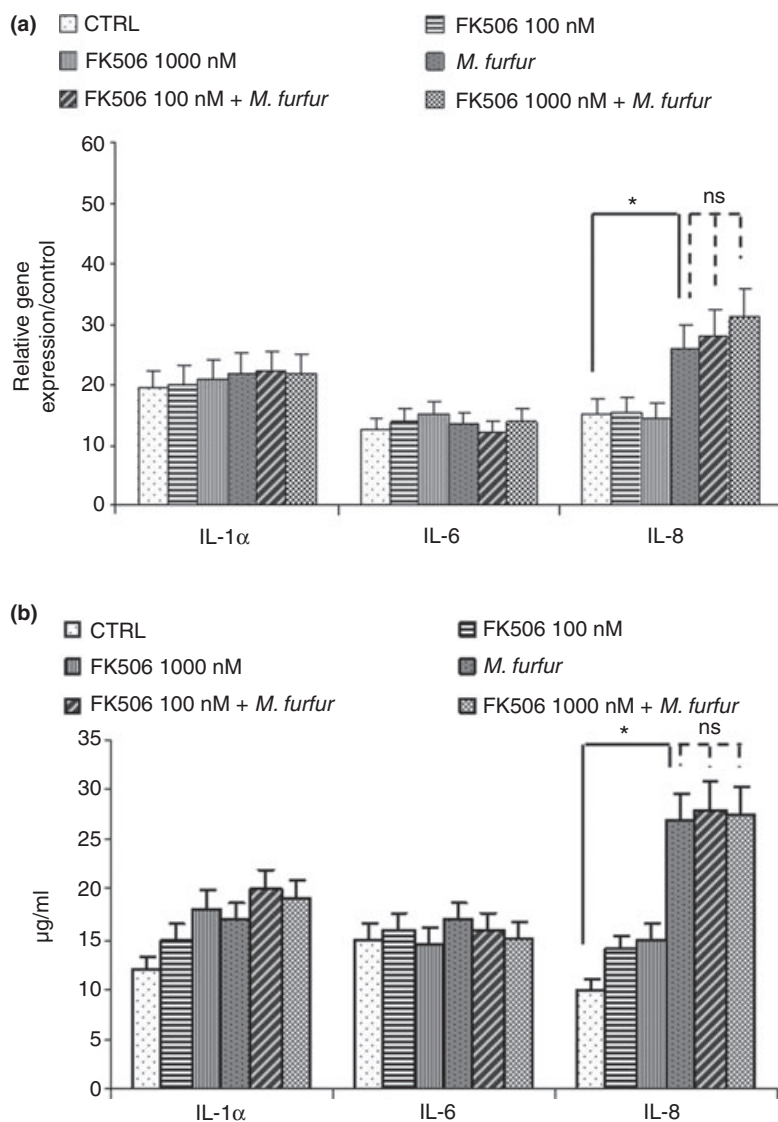


Figure 3 Effects of FK506 on IL-1 α , IL-6 and IL-8 in human keratinocytes (KCs) infected with *Malassezia furfur*. (a) IL-1 α , IL-6 and IL-8 gene expression in KCs at 48 h after treatments; values are expressed as mRNA relative to the house-keeping gene β -actin. (b) ELISA assay. IL-1 α , IL-6 and IL-8 release from KCs at 72 h after treatments. Data are displayed as mean $\pm$ standard deviation of five independent experiments, each performed in duplicate. The ANOVA test (analysis of variance between groups) was performed for each kind of experiment. ns, Not significant; * $P < 0.05$.

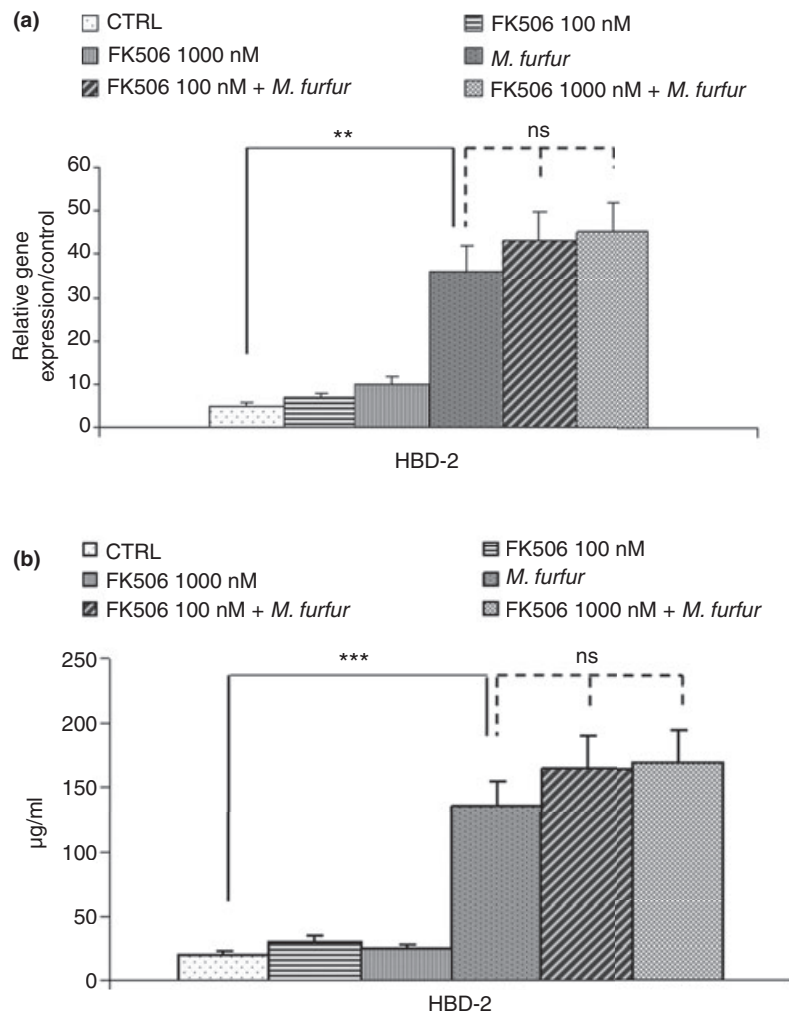


Figure 4 Effects of FK506 on human β -defensin-2 (HBD-2) in human keratinocytes (KCs) infected with *Malassezia furfur*. (a) HBD-2 gene expression in KCs at 48 h after treatments; values are expressed as mRNA relative to the house-keeping gene β -actin. (b) ELISA assay. HBD-2 release from KCs at 72 h after treatments. Data are displayed as mean $\pm$ standard deviation of five independent experiments, each performed in duplicate. The ANOVA test (analysis of variance between groups) was performed for each kind of experiment. ns. Not significant; ** $P < 0.01$; *** $P < 0.001$.

TGF- β 1 allowed us to hypothesise a possible anti-proliferative effect. This concept has already been reported, showing that the anti-proliferative effect of FK506 directly correlated with blockade of KCs cell cycle at the G0/G1 phases.³⁵ This finding may underneath the therapeutical success in proliferative skin disorders, like psoriasis. The anti-inflammatory cytokine IL-10 overproduction, induced by *M. furfur*, was not suppressed by FK506 treatment, indicating that IL-10 is free to act. *M. furfur* could not affect IL-1 α and IL-6 expression and production. This finding was in line with Watanabe *et al.* [36] which showed that *M. furfur* did not induce an increase in these cytokines in KCs. Neither FK506 altered the production of these cytokines, suggesting that it has no an immune suppressant activity.

IL-8 is a pro-inflammatory cytokine that belongs to the chemokine family and has a direct effect on immune cells; it is a powerful chemoattractant for

neutrophils, basophils and T cells.³⁷ In this study, FK506 was not able to suppress the overproduction of IL-8 induced by *M. furfur*, so we can hypothesise that the high level of IL-8 could provide the integrity of immune responses, even after mycotic stimulus. Regarding AMPs, our data showed that FK506 was not able to induce HBD-2 expression and production in KCs. Moreover, FK506 could not suppress the enhanced expression of HBD-2 induced by *M. furfur*, suggesting that its use may not render susceptibility to infections. Buchau *et al.* [38] investigated the effects of pimecrolimus on pro-inflammatory cytokines and AMPs in human KCs. They found that pimecrolimus treatment only did not interfere with IL-1 β , IL-6, IL-8 and IL-10 expression with respect to normal KCs, supporting our results. Furthermore, they also showed that pimecrolimus suppressed the expression of IL-1 β and IL-10 when stimulated by Malp-2 (toll-like receptors 2/6-dependent microbial stimulus), whereas

FK506 and cyclosporin A did not inhibit IL-6 and IL-8 expression. Kis *et al.* [29] reported that FK506 treatment only did not interfere with IL-1 α and IL-8 expression with respect to normal KCs, being in line with our findings.

Immunosuppressants used topically in several inflammatory skin diseases can not only influence the function of professional immune cells (e.g. lymphocytes) infiltrating the skin but they can also affect the immune functions of KCs.³⁹ Because in chronic inflammatory skin disorders, differential expression of AMPs appears to play a role in susceptibility to infectious complications, our results about HBD-2 suggest that FK506 may represent a therapeutical option with relative safety regarding infections.

In conclusion, prolonged use of topical immunosuppressants may exacerbate cutaneous infections and this adverse effect might be partly due to an inhibitory effect on KCs cytokine production. We found that, after infection with *M. furfur*, the production by KCs of several cytokines (IL-1 α , IL-6, IL-8, IL-10, TGF- β 1) as well as of the AMP HBD-2 was not influenced by FK506; this confirmed its anti-inflammatory activity and could lead to the hypothesis that this drug may not affect the risk of mycotic skin infection when used in different inflammatory skin disorders.

Conflict of interest

The authors declare that they have no conflict of interest.

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