**Informative title:**

**Responses of reconstructed human epidermis to *Trichophyton rubrum* infection and impairment of infection by the inhibitor PD169316**

**Short title:**

**Dermatophytosis inhibition by PD169316**

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**Abbreviations used:** AMP, antimicrobial peptide; Cldn-1, claudin-1; CN, copy number; hBD, human β-defensin; LY, Lucifer Yellow; RHE, reconstructed human epidermis; SC, *stratum corneum*; TEER, trans-epithelial electrical resistance; TSG-6, TNFα-stimulated gene 6; TJ, tight junction.

# ABSTRACT

Despite threatening incidence of dermatophytosis, information is still lacking about the consequences of infection onto epidermal barrier functions and about keratinocyte responses devoted to alert immune components. In order to identify involved mechanisms, arthroconidia of *Trichophyton rubrum* an anthropophilic dermatophyte were prepared to infect reconstructed human epidermis (RHE) *in vitro*. Integrity of the barrier was monitored during infection by measurements of trans-epithelial electrical resistance and dye-permeation through the RHE. Expression and release of pro-inflammatory cytokines and antimicrobial peptides (AMP) by keratinocytes inserted in RHE were respectively assessed by RT-qPCR to analyze mRNA content in tissue extracts and by ELISA to detect proteins in culture media. Results reveal that infection by *T. rubrum* is responsible for disruption of the epidermal barrier, including loss of functional tight junctions, and for simultaneous expression and release of cytokines and AMP by keratinocytes. Potential involvement of the p38 mitogen-activated protein kinase (MAPK) signaling pathway was evaluated during infection by targeted inhibition of its activity. Intriguingly, among several p38 MAPK inhibitors, PD169316 alone was able to inhibit growth of *T. rubrum* on Sabouraud agar and to suppress the process of infection on RHE, suggesting that PD169316 acts on specific target in dermatophytes themselves.

# INTRODUCTION

Dermatophytosis is a superficial mycosis whose prevalence is estimated between 20 and 25% in the global human population and is increasing since the last decade (Havlickova et al., 2008; Seebacher et al., 2008; Hayette et al., 2015; Zhan et al., 2017). The anthropophilic *Trichophyton rubrum* species is the most common dermatophyte responsible for glabrous skin infection (Tomoyuki et al., 2014; Lee et al., 2015), which are generally limited to the *stratum corneum* (SC) (Weitzman et al., 1995; ~~Squeo et al., 1998; Cheng et al., 2014~~). The confinement of fungal hyphae and spores in superficial epidermal layers is thought to be associated with both the epidermal barrier itself and the appropriate activation of the immune system (Mignon et al., 2008; Vermout et al., 2008; Martinez-Rossi et al., 2017). The latter is known to induce not only innate immune responses, but also an adaptive immune response involving TCR-mediated immunity, that is critical for fungal clearance and clinical recovery (Calderon and Hay, 1984; Burstein et al., 2018; Heinen et al., 2018). However, consequences of dermatophytic infection onto epidermal barrier and keratinocyte responses alerting innate immune components remain poorly understood.

The epidermal barrier protects the organism against external aggressions and water loss (Bäsler et al., 2016). Its efficiency is provided by the collaboration between physical components, i.e. SC and tight junctions (TJ), antimicrobial peptides (AMP), cells of the immune system and the skin microbiome (Proksch et al., 2008;Brandner et al., 2016). SC, composed of corneocytes~~, filled with keratin aggregates, lined by the cornified envelope, and~~ maintained together by corneodesmosomes across intercellular lipid matrix, is extremely resistant to physical stress and relatively impermeable to water and many chemicals (Haftek et al., 2015; van Smeden et al., 2016). TJ are intercellular junctions established between granular keratinocytes and are responsible for the paracellular impermeability in the epidermis (Kirschner et al., 2013).

In case of infection, keratinocytes are the first cells to encounter dermatophytes. They can detect pathogens via pathogen-associated molecular pattern recognition by pattern recognition receptors, notably TLR (toll-like receptor) 2, 4 and 6 ~~involved in dermatophytes recognition~~ (Garcia-Madrid et al., 2011; Brasch et al., 2014; Cambier et al., 2016). Activation of pattern recognition receptors on keratinocytes induces signaling pathways leading to expression and release of pro-inflammatory cytokines and AMP. Notably, the p38 MAPK (mitogen-activated protein kinase) signaling pathway can be activated in response to environmental stresses and is implied in a variety of cellular processes including inflammation (Zaburin et al,. 2005 ; Kyriakis et al., 2012). Interestingly, the p38 MAPK signaling pathways was involved in AMP expression by keratinocytes exposed to a cell wall component of *Candida albicans* (Li et al., 2011) and was found activated in keratinocytes exposed to *Trichophyton equinum* dermatophytes (Achterman et al., 2015). Besides, several studies performed *in vitro* on keratinocytes monolayers have shown increased expression and release of cytokines, such as TNFα, IL-1α, IL-1β, IL-6, IL-8, *etc.*, in response to stimulation by dermatophytes (Nakamura et al., 2002; Shiraki et al., 2006; Tani et al., 2007). However, identities of detected cytokines considerably vary depending on the dermatophytes species, zoophilic species generally inducing more inflammatory lesions than anthropophilic ones.

In addition, the exposure of keratinocytes cultured as monolayers to fungal cells is poorly representative of *in vivo* infection. *In vivo* models of dermatophytosis using guinea pig (Baldo et al. 2010) or mouse (Cambier et al., 2014; Heinen et al., 2018) are useful to study the immune responses against dermatophytosis but differences could exist with human infection. Therefore, culture of human skin equivalents appear as quite relevant models to study human dermatophytosis.

Such a model of dermatophytosis was setup through infection of reconstructed human epidermis (RHE) by arthroconidia of the anthropophilic *T. rubrum* species (Faway et al., 2017). This model appears rather representative of human dermatophytosis, with fungal components proliferating over time~~, as demonstrated by histological analysis and PCR quantification,~~ and invading the SC. Herein, this model has been used in order to assess damages to the epidermal barrier, as well as to detect specific activation of keratinocyte responses during infection. While investigating the potential involvement of p38 MAPK signaling pathway in the reported keratinocyte responses to infection, unique effects of PD169316 compound, a well-known inhibitor of p38 MAPK, were highlighted on growth and infection properties of dermatophytes.

# RESULTS

## Fungal hyphae invade the SC by progressing between corneocytes

Progression of fungal elements through RHE infected by *T. rubrum* arthroconidia was monitored over time by morphological analysis (**Figure 1a**). A progressive invasion into SC was observed: on the first day, hyphae emerging from arthroconidia spread over the SC surface and penetrated SC by sneaking between corneocytes (**Figure 1b**); on the fourth day, hyphae are found in intercellular space through the full thickness of SC (**Figure 1c**).

## *T. rubrum* infection simultaneously alters integrity of the epidermal barrier and activates keratinocyte responses

The epidermal barrier integrity of RHE was assessed during infection by measurement of trans-epithelial electrical resistance (TEER) and permeability to Lucifer Yellow (LY) fluorescent dye (**Figure 2 a-c**). Sudden barrier alterations appear on the fourth day following infection. Conversely, the barrier is strengthened over time in non-infected RHE. Additionally, inside-out permeability of RHE was assessed using biotin dissolved in culture medium, revealing that the integrity of TJ is reduced on the fourth day after infection (**Figure 2d**). Accordingly, the TJ protein claudin-1 (Cldn-1) exhibits an altered distribution in infected RHE as assessed by immunostaining (**Figure S1**).

Infection of RHE further triggers mRNA expression in keratinocytes, as well as cell release, of cytokines (IL-8, IL-1α, IL-1β, TNFα, TSLP, G-CSF), of protein TNFα-stimulated gene 6 (TSG-6) and AMP (human β-defensins -2 (hBD2) and -3 (hBD3), S100A7) as respectively revealed by RT-qPCR and ELISA (**Figure 2 e-f** and **Figure S2**).Conversely, except for transglutaminase-1 which is slightly overexpressed one day after infection, the expression of differentiation markers (filaggrin, involucrin or loricrin) or of TLR2, 5 and 6 exhibits no alteration during infection (**Figure S3**). Expression and release of all factors studied remained unaltered inside RHE exposed to phosphate-buffered saline (PBS) alone or to heat-killed arthroconidia.

The copy number (CN) for the DEFB4 gene, encoding hBD2, can range from 2 to 12 and is linked to variations in basal expression levels for hBD2 (Hollox et al., 2003). High CN in patients affords reduced susceptibility to dermatophytosis (Jaradat et al., 2015). The most frequent DEFB4 CN in population being 4, CN under 4 are considered “low” and those over 4 as “high” (Jaradat et al., 2013). Eight primary keratinocytes cultured in our laboratory were genotyped to count DEFB4 CN. Data reveal between 3 and 5 CN for this gene (**Figure S4**). Keratinocytes with 3 DEFB4 CN were selected for RHE production in this study.

## p38 MAPK inhibitor PD169316 hampers infection of RHE by affecting dermatophytes growth

Because activation of p38 MAPK has been associated with infection by dermatophytes (Achterman et al., 2015) and is also found in untreated RHE (**Figure 3**), potential role for p38 MAPK during *T. rubrum* infection of RHE was investigated by use of PD169316, a p38 MAPK specific inhibitor. No further activation of p38 MAPK can be observed during RHE infection (**Figure 3a**). Thus, the actual inhibition of p38 MAPK activity by PD169316 was assessed through detection of heat shock protein 27 (HSP27) phosphorylation, a known ~~phosphorylation~~ target of p38 MAPK in keratinocytes (Garmyn et al., 2001), in RHE exposed to H2O2 treatment in order to activate p38 MAPK signaling pathway (Peus et al., 1999; Mathay et al., 2008) (**Figure 3b**).

Interestingly, the presence of PD169316 prevents the epidermal barrier alterations usually induced on the fourth day after *T. rubrum* infection **(Figure 4a-c**). Besides, the extent of SC invasion by arthroconidia is also reduced (**Figure 4d**)whereas the overexpression of IL-8, IL-1α, IL-1β, TNFα, hBD2 and hBD3 usually induced by infection is not observed in presence of PD169316 (**Figure 4e** and **Figure S5**).

Because p38 MAPK activity seems unaltered during infection of RHE, a potential effect of PD169316 on *T. rubrum* growth itself was hypothesized and studied by seeding arthroconidia on Sabouraud agar (2% glucose and 1% peptone) containing PD169316. After seven days incubation at 27°C, the number of colony-forming units is reduced in presence of PD169316 and colonies appears smaller and more compact, while fungal hyphae seem thinner and present less septa when analyzed through scanning electron microscopy (**Figure 5a-b**). In good accordance with an effect of PD169316 on dermatophytes, growth of *T. rubrum* arthroconidia on lyophilized RHE was impaired by PD169316 (**Figure 5c**). Similar growth inhibition is observed for two other species, *Trichophyton interdigitale* and *Trichophyton benhamiae*, seeded on Sabouraud agar containing PD169316 (**Figure 5d-e**). Conversely though, other p38 MAPK inhibitors, namely SB202190, SB203580, VX-702 and BIRB796, do not alter growth of *T. rubrum* (**Figure 6**), *T. interdigitale* or *T. benhamiae* (**Figure S6**) on Sabouraud agar, nor the infection of RHE by *T. rubrum* arthroconidia (data not shown). Besides, culture in presence of PD169316 or SB203580 does not suppress growth of the fission yeast *Schizosaccharomyces pombe*, whereas strain knockout for *sty1*, the yeast homolog for p38 MAPK, exhibits drastically reduced cell growth in any culture condition (**Figure S7**).

# DISCUSSION

The *in vitro* model of *T. rubrum* dermatophytosis in RHE has been previously validated as representative of *in vivo* human cutaneous dermatophytosis lesions (Faway et al., 2017). Here, the consequences of infection on epidermal barrier integrity, and the primary responses of keratinocytes are characterized.

**Hyphae of *T. rubrum* invade *stratum corneum* and disrupt the epidermal barrier**

Electron microscopy images suggest that *T. rubrum* hyphae invade the SC by intercellular progression between corneocytes. Accordingly, hyphae of *Trichophyton mentagrophytes* were previously shown to invade the SC between corneocytes both in infected SC sheets (Aljabre et al., 1992) and skin sections (Duek et al., 2004). These results suggest that dermatophytes are able to orientate the direction of hyphal growth in relation to the physical and topographical features of the substrate, a phenomenon known as “thigmotropism” regulated by complex molecular signaling pathways (Almeida et al., 2017). Thigmotropism is required *in vitro* and *in vivo* for invasion of host surface by pathogenic fungi such as *C. albicans* and dermatophytes (Perera et al., 1997; Jayatilake et al., 2005; Piérard et al., 2007). These observations also suggest that dermatophytes may degrade corneodesmosomes and parts of the lipid extracellular matrix while hyphae invade the SC. As dermatophytes secrete several proteases during *in vivo* infection (Méhul et al., 2016; Tran et al., 2016), hyphae may thus be responsible for altering barrier integrity observed during infection of RHE.

In absence of immune system and microbiome, the RHE barrier relies on physical components of SC and TJ (Proksh et al., 2008), explaining that it strengthens over time in untreated RHE as a likely result of SCthickening. Barrier function of RHE temporarily covered with PBS increases similarly, suggesting that transient moistening of the epidermal surface does not alter barrier integrity. In contrast, the barrier of infected RHE is suddenly disrupted on the fourth day following infection by *T. rubrum* arthroconidia, in line with an observed increased trans-epithelial water loss in biopsies from dermatophytosis cutaneous lesions (Jensen et al., 2007). In addition, the function of TJ is lost on the fourth day after RHE infection, which could be partly explained by the altered localization of Cldn-1. Similarly, perturbed TJ have already been observed *in vitro* and *in vivo* during *Staphyloccocus aureus* infection and found associated to a simultaneous redistribution of proteins from TJ (Ohnemus et al., 2008; Bäsler et al., 2017). This barrier disruption allows dermatophytes to colonize the full thickness of RHE in the absence of immune cells. Further evidence is provided by histological demonstration that fungal elements invade living cell layers of RHE from the fifth day following infection (Faway et al., 2017). Of note, mRNA expression of epidermal differentiation markers remains unaltered during *T. rubrum* infection of RHE, even though *T. rubrum* infection disturbs epidermal morphology. This observation does not exclude concomitant redistribution of differentiation markers, as previously observed in dermatophytosis lesions (Jensen et al., 2007).

In this study, we used keratinocytes strains with low DEFB4 CN to produce RHE. Since high DEFB4 CN is associated with reduced susceptibility to develop dermatophytosis in patients (Jaradat et al., 2015), it would be interesting to compare infection of RHE produced from keratinocytes strains with low or high DEFB4 CN.

***T. rubrum* infection induces inflammatory responses in RHE**

Keratinocytes of infected RHE overexpress and release several cytokines (IL-1α, IL-1β, TNFα, IL-8) and AMP (hBD2, hBD3, S100A7) from the fourth day after infection. ~~As an exception, TSLP is overexpressed on the first day, possibly representing an early marker of~~ *~~T. rubrum~~* ~~infection.~~ Release of G-CSF, an early response of epithelial cells to *Candida albicans* infection (Moyes et al., 2016), was detected at low concentration in our model, whilst IL-6 and RNase7 expression is scarcely detectable. In contrast, neither transient moistening of the tissue, nor exposition to killed arthroconidia were able to induce those responses. Some *in vitro* studies report release of these cytokines by infected keratinocytes, while others do not (Nakamura et al., 2002; Shiraki et al., 2006; Tani et al., 2007; Achterman et al., 2015). Expression of pro-inflammatory cytokines and subsequent activation of Th1 and Th17 immunity have been observed in a mouse model of dermatophytosis (Cambier et al., 2014; Heinen et al., 2018). Furthermore, overexpression and release of hBD2, hBD3, RNase7 and S100A7 in response to dermatophyteshave been demonstrated *in vitro* (Firat et al., 2014) and *in vivo* (Brasch et al., 2014). The apparent discrepancies in cytokine profiles may represent distinct keratinocyte responses to different dermatophytes species. In line with this, specific cytokines profiles characterize the responses to anthropophilic versus zoophilic dermatophytes species (Shiraki et al., 2006; Tani et al., 2007). In addition to inflammatory cytokines, TSG-6, an anti-inflammatory protein, was produced in response to *T. rubrum* infection of RHE. Accordingly, infection by *T. rubrum* induces the release of the anti-inflammatory cytokine IL-10 by macrophages (Campos et al., 2006).

The simultaneous activation of keratinocyte responses and epidermal barrier disruption suggests that both events are related. Perturbation of the epidermal barrier allows contact between dermatophytes and living granular keratinocytes able to recognize fungal molecular patterns through TLR (Netea et al., 2008), inducing production of cytokines and AMP. However, a reverse mechanism cannot be excluded: during invasion of SC by dermatophytes, granular keratinocytes could recognize fungal secreted material (Brouta et al., 2003; Descamps et al., 2003) and react by producing cytokines and AMP which can in turn influence the epidermal barrier (Kirschner et al., 2009).

**PD169316 inhibits growth of dermatophytes**

As p38 MAPK activation had been previously associated with dermatophyte infection (Achterman et al., 2015) ~~and alterations of TJ barrier (Kanemaru et al., 2017)~~, its role during infection of RHE was monitored by using PD169316, a p38 MAPK specific inhibitor (Jans et al., 2004; Mathay et al., 2008). Unexpectedly, PD169316 was shown to inhibit growth of arthroconidia from *T. rubrum*, *T. interdigitale* and *T. benhamiae*, both on RHE and on Sabouraud agar. Moreover, colonies grown *in vitro* in presence of PD169316 are smaller and display abnormal morphology. ~~Intriguingly, similar morphological anomalies of~~ *~~T. rubrum~~* ~~hyphae have been reported upon treatment of fungi with synthetic azole and upon photodynamic treatment with a synthetic porphyrin photosensitizer (Mares et al., 1998; Smijs et al., 2008).~~

A candidate fungal target of PD169316 is a *T. rubrum* p38 protein kinase ortholog with 50% aminoacid identity to human p38~~ and p38 the most abundant isoforms of p38 MAPK expressed in keratinocytes (Dashti et al., 2001)~~. ~~Interestingly and in accordance with a primordial role for MAPK in other fungal infection, a crucial MAPK activity required for invasion of plant tissue was recently identified in the filamentous fungus~~ *~~Magnaporthe oryzae~~*~~, responsible for disease of cereals (Sakulkoo et al., 2018). This observation raises the need for improving understanding of dermatophyte biology in order to rationally design new prophylactic treatments against dermatophytosis~~. However, PD169316 does not alter growth of yeast *S. pombe* whose p38 MAPK ortholog Sty1 shares 84% identity with *T. rubrum* p38 protein kinase. This observation suggests that the fungal target of PD169316, whose inhibition leads to dermatophytes growth impairment, could not be the p38 protein kinase. In addition, other p38 MAPK inhibitors SB202190, SB203580, VX-702 and BIRB796, do not inhibit dermatophytes growth on Sabouraud agar, nor infection of RHE. Thus, among p38 MAPK inhibitors, PD169316 exhibits unique effects as observed in a study investigating antiviral activities against Enterovirus71 (Zhang et al., 2017).

**Summary**

The present report illustrates that *T. rubrum* infection of RHE results into simultaneous epidermal barrier disruption and activation of keratinocyte responses. It shows that PD169316 inhibits dermatophyte growth, thereby qualifying as a novel candidate drug against dermatophytosis.

# MATERIALS & METHODS

## Dermatophyte strains, culture and production of arthroconidia

The strains IHEM 13894 of *T.* *rubrum*, IHEM 00584 of *T. interdigitale* and IHEM 20163 of *T. benhamiae*, isolated from naturally infected human, were obtained from the Belgian Co-ordinated Collections of Micro-organisms (BCCM/IHEM collection of biomedical fungi and yeasts, Brussels, Belgium). Arthroconidia were produced as previously described (Tabart et al., 2007). Briefly, dermatophytes grown at 27°C on Sabouraud agar (containing 2% glucose and 1% peptone) were recovered and cultured on 2% yeast extract 1% peptone agar at 30°C in an atmosphere containing 2% CO2. Surface mycelium was scraped, added to sterile PBS and this solution was stirred overnight at 4°C and then filtered through three Miracloth layers (22-25 µm pore size; Millipore, Overijse, Belgium) in order to recover unicellular fungal elements corresponding to arthroconidia. Heat-killed arthroconidia were obtained by 10 min incubation in a boiling water bath followed by PBS washes.

## Infection of reconstructed human epidermis by *T. rubrum* dermatophyte

RHE were prepared as described (De Vuyst et al., 2014) and cultured in EpiLife medium (Cascade Biologics, Mansfield, UK) supplemented with Human Keratinocyte Growth Supplement (Cascade Biologics, Mansfield, UK) and containing 1.5 mM Ca2+, 10 ng/ml keratinocyte growth factor (R&D system, Abingdon, UK) and 50 µg/ml vitamin C.

The procedure used for infection was previously described (Faway et al., 2017). Concisely, RHE were infected on day 0 (D0) by topical application of a PBS-suspension of *T. rubrum* arthroconidia in order to reach a density of 1,700 arthroconidia per cm². Four hours later, the suspension was removed from RHE, followed by washes with PBS. Infected RHE were then cultured at 37°C in a humidified atmosphere containing 5% CO2 for four additional days with culture medium renewed every day.

PBS-treated RHE consisted in RHE on which only PBS was applied on D0 and which have undergone washes. For infection of RHE with heat-killed arthroconidia, PBS was first topically applied on D0, followed four hours later by washes. On the fourth day following PBS-exposure, 1,000,000 heat-killed *T. rubrum* arthroconidia were topically applied and infected RHE were finally analyzed after four hours of incubation.

## Histological analysis

For histological analysis, RHE were processed as described (De Vuyst et al., 2014). Periodic-acid Schiff (PAS) staining was performed with hemalun counterstaining and pretreatment with α-amylase as previously described (Faway et al., 2017).

## Assessment of epidermal barrier integrity

TEER of RHE was measured using a Millicell® ERS-2 volt-ohm meter (Millipore, Overijse, Belgium) and expressed as percentage of values determined in control RHE.

To assess the permeability of RHE, 150 µl of fluorescent dye (Lucifer Yellow Vs dilithium salt, Sigma-Aldrich, Munich, Germany) were laid on the surface of RHE placed over 200 µl of culture medium. RHE were incubated at 37°C for 6 hours in a humidified atmosphere containing 5% CO2*,*protected from light. The amount of LY in the medium under the RHE was finally determined by measuring its fluorescence using a fluorescence reader. In addition, sections of RHE were observed using a fluorescent microscope in order to localize LY in tissue.

To investigate efficiency of the inside-out barrier, biotin diffusion from culture media to the apical surface of RHE was assessed. After washes with PBS containing 1 mM CaCl2, RHE were incubated at 37°C in a humidified atmosphere containing 5% CO2 for 30 min, over 500 µl of the same solution containing 2 mg/ml biotin (EZ-LinkTM Sulfo-NHS-LC-Biotin; ThermoScientific, Rockford, IL). RHE were washed with PBS containing CaCl2 and 100 mM glycine, fixed for 24h in 4% formaldehyde solution and finally embedded in paraffin. Tissue sections were stained with streptavidin conjugated to Alexa Fluor® 488 (dilution 1:500; Invitrogen, Aalst, Belgium) and the localization of biotin was observed using a fluorescence microscope.

## Immunostaining, electron microscopy, RNA extraction and RT-qPCR, ELISA and Western blot

For detailed description of immunofluorescence staining of Cldn-1, of scanning and transmission electron microscopy, of total RNA extraction, reverse-transcription and quantitative PCR, of ELISA and of protein extraction and Western blot analysis, see the Supplementary Materials and Methods.

## p38 MAPK Inhibitors

BIRB796 was purchased from Tocris (Abingdon, UK), PD169316 from Santa Cruz biotechnology (Heidelberg, Germany), SB202190 and SB203580 from InvivoGen (Toulouse, France) and VX-702 from Selleckchem (Munich, Germany). Concentration of each inhibitor was 15 µM.

In order to study the efficiency of PD169316, p38 MAPK signaling pathway was stimulated by treating RHE during 20 or 60 min with 1 mM H2O2 added to the culture media, followed by one hour of recovery. PD169316 was present in culture media of RHE 24h before addition of H2O2 and during treatment and recovery.

## Statistical Analyses

All statistical analyses were carried out using SigmaPlot 11.0 software. One-way analysis of variance (ANOVA1) and *t*-Student test were performed to analyze our data.

# CONFLICT OF INTEREST

The authors state no conflict of interest.

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# REFERENCES

Achterman R, Moyes D, Thavaraj S, Smith A, Blair K, White T, et al. Dermatophytes activate skin keratinocytes via mitogen-activated protein kinase signaling and induce immune responses. Infect Immun 2015;83:1705-1714.

Aljabre S, Richardson M, Scott E, Shankland G. Germination of *Trichophyton mentagrophytes* on human stratum corneum *in vitro*. J Med Vet Mycol 1992;30:145-152.

Almeida M, Brand A. Thigmo responses: the fungal sens of touch. Microbiol Spectr 2017;5:FUNK-0040-2016.

Baldo A, Mathy A, Tabart J, Camponova P, Vermout S, Massart L, et al. Secreted subtilisin Sub3 from *Microsporum canis* is required for adherence to but not for invasion of the epidermis. Br J Dermatol 2010;162:990-997.

Bäsler K, Bergmann S, Heisig M, Naegel A, Zorn-Kruppa M, Brandner J. The role of tight junctions in skin barrier function and dermal absorption. J Control Release 2016;242:105-118.

Bäsler K, Galliano M-F, Bergmann S, Rohde H, Wladykowski E, Vidal-y-Sy S, et al. Biphasic influence of *Staphylococcus aureus* on human epidermal tight junctions. Ann N Y Acad Sci 2017;1405;53-70.

Brandner J. Importance of tight junctions in relation to skin barrier function. Curr Probl Dermatol 2016;49:27-37.

Brasch J, Mörig A, Neumann B, Proksch E. Expression of antimicrobial peptides and toll-like receptors is increased in *tinea* and *pityriasis versicolor*. Mycoses 2014;57:147-152.

Brouta F, Descamps F, Vermout S, Monod M, Losson B, Mignon B. Humoral and cellular immune response to a *Microsporum canis* recombinant keratinolytic metalloprotease (r-MEP3) in experimentally infected guinea pigs. Med Mycol 2003;41;495-502.

Burstein VL, Guasconi L, Beccacece I, Theumer MG, Mena C, Prinz I, et al. IL-17-mediated immunity controls skin infection and T helper 1 response during experimental *Microsporum canis* dermatophytosis. J Invest Dermatol 2018;138:1744-1753.

Calderon RA, Hay RJ. Cell-mediated immunity in experimental murine dermatophytosis. II. Adoptive transfer of immunity to dermatophyte infection by lymphoid cells from donors with acute or chronic infections. Immunology 1984;53:465-472.

Cambier L, Weatherspoon A, Defaweux V, Bagut E, Heinen M, Antoine N, et al. Assessment of the cutaneous response during *Arthroderma benhamiae* and *A. vanbreuseghemii* infection using an experimental mouse model. Br J Dermatol 2014;170:625-633.

Cambier L, Heinen M, Bagut E, Antoine N, Mignon B. Overexpression of TLR-2 and TLR-4 mRNA in feline polymorphonuclear neutrophils exposed to *Microsporum canis*. Vet Dermatol 2016;27:78-e22.

Campos M, Russo M, Gomes E, Almeida S. Stimulation, inhibition and death of macrophages infected with *Trichophyton rubrum*. Microbes Infect 2006;8:372-379.

Cheng C, Hsiao Y, Ko. J. Disseminated deep dermatophytosis caused by *Trichophyton rubrum*. Dermatol Sin 2014;32:191-192.

Dashti S, Efimova T, Eckert R. MEK7-dependent activation of p38 MAP kinase in keratinocytes. J Biol Chem 2001;276:8059-8063.

De Vuyst E, Charlier C, Giltaire S, De Glas V, Lambert de Rouvroit C, Poumay Y. Reconstruction of normal and pathological human epidermis on polycarbonate filter. Methods Mol Biol.2014;1195:191-201.

Descamps F, Brouta F, Vermout S, Monod M, Losson B, Mignon B. Recombinant expression and antigenic properties of a 31.5 kDa keratinolytic subtilisin-like serine protease from *Microsporum canis*. FEMS Immunol Med Microbiol 2003;38:29-34.

Duek L, Kaufman G, Ulman Y, Berdicevsky I. The pathogenesis of dermatophyte infection in human skin sections. J Infect 2004;48:175-180.

Faway E, Cambier L, Mignon B, Poumay Y, Lambert de Rouvroit C. Modeling dermatophytosis in reconstructed human epidermis: A new tool to study infection mechanisms and to test antifungal agents. Med Mycol 2017;55:485-494.

Firat Y, Simanski M, Rademacher F, Schröder L, Brasch J, Harder J. Infection of keratinocytes with *Trichophyton rubrum* induces epidermal growth factor-dependent RNase 7 and human beta-defensin-3. PLos ONE 2014;9:e93941.

Garcia-Madrid L, Huizar-Lopez M, Flores-Romo L, Islas-Rodriguez A. *Trichophyton rubrum* manipulates the innate immune functions of human keratinocytes. Cent Eur J Biol 2011;6:902-910.

Garmyn M, Mammone T, Pupe A, Gan D, Declercq L, Maes D. Human keratinocytes respond to osmotic stress by p38 MAP kinase regulated induction of HSP70 and HSP27. J Invest Dermatol 2001;117:1290-1295.

Haftek M. Epidermal barrier disorders and corneodesmosome defects. Cell Tissue Res 2015;360:483-490.

Havlickova B, Czaika V, Friedrich M. Epidemiological trends in skin mycoses worldwide. Mycoses 2008;51:2-15.

Hayette M, Sacheli R. Dermatophytosis, trends in epidemiology and diagnostic approach. Curr Fungal Infect Rep 2015;9:164-179.

Heinen M, Cambier L, Antoine N, Gabril A, Gillet L, Bureau F, et al. Th1 and Th17 immune responses act complementary to optimally control superficial dermatophytosis. J Invest Dermatol 2018;139:626-637.

Hollox EJ, Armour JA, Barber JC. Extensive normal copy number variation of a β–defensin antimicrobial-gene cluster. Am J Hum Genet 2003;73:591-600.

Jans R, Atanasova G, Jadot M, Poumay Y. Cholesterol depletion upregulates involucrine expression in epidermal keratinocytes through activation of p38. J Invest Dermatol 2004;123:564-573.

Jaradat SW, Hoder-Przyrembel C, Cubillos S, Krieg N, Lehmann K, Piehler S, et al. Beta-defentin-2 genomic copy number variation and chronic periodontitis. J Dent Res 2013;92:1035-1040.

Jaradat SW, Cubillos S, Krieg N, Lehmann K, Issa B, Piehler S, et al. Low DEFB4 copy number and high systemic hBD-2 and IL-22 levels are associated with dermatophytosis. J Invest Dermatol 2015;135:750-758.

Jayatilake J, Samaranayake Y, Samanarayake L. An ultrastructural and a cytochemical study of candida invasion of reconstructed human oral epithelium. J Oral Pathol Med 2005;34:240-246.

Jensen J, Pfeiffer S, Akaki T, Schröder J, Kleine M, Neumann C, et al. Barrier function, epidermal differentiation, and human β-defensin 2 expression in *tinea corporis*. J Invest Dermatol 2007;127:1720-1727.

~~Kanemaru K, Nakamura Y, Totoki K Fukuyama T, Shoji M, Kaneko H, et al. Phospholipase Cδ1 regulates p38 MAPK activity and skin barrier integrity, Cell Death Differ 2017;24:1079-1090.~~

Kirschner N, Poetzl C, von den Drieschn P, Wladykowski E, Moll I, Behne M, et al. Alteration of tight junction proteins is an early event in psoriasis: putative involvement of proinflammatory cytokines. Am J Pathol 2009;175:1095-1106.

Kirschner N, Rosenthal R, Furuse M, Moll I, Fromm M, Brandner J. Contribution of tight junction proteins to ion, macromolecule, and water barrier in keratinocytes. J Invest Dermatol 2013;133:1161-1169.

Kyriakis JM, Avruch J. Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update. Physiol Rev 2012;92:689-737.

Lee W, Kim S, Jang Y, Lee S, Kim D, Bang Y, et al. Increasing prevalence of *Trichophyton rubrum* identified through an analysis of 115,846 cases over the last 37 years. J Korean Med Sci 2015;30:639-643.

Li M, Chen Q, Tang R, Shen Y, Liu WD. The expression of β-defensin-2, 3 and LL-37 induced by *Candida albicans* phospholipomannan in human keratinocytes. J Dermatol Sci 2011;61:72-75.

Mares D, Romangnoli C, Sacchetti G, Vicentini C, Bruni A. Morphological study of *Trichophyton rubrum*: ultrastructural findings after treatment with 4-amino-3-methyl-l-phenylpyrazolo-(3,4-c)isothiazole. Med Mycol 1998;36:379-385.

Martinez-Rossi NM, Peres NT, Rossi A. Pathogenesis of dermatophytosis: sensing the host tissue. Mycopathologia 2017;182:215-227.

Mathay C, Giltaire S, Minner F, Bera E, Hérin M, Poumay Y. Heparin-binding EGF-like growth factor is induced by disruption of lipid rafts and oxidative stress in keratinocytes and participates in the epidermal response to cutaneous wounds. J Invest Dermatol 2008;128:717-727.

Méhul B, Gu Z, Jomard A, Laffet G, Feuilhade M, Monod M. Sub6 (Tri r 2), an onychomycosis marker revealed by proteomics analysis of *Trichophyton rubrum* secreted proteins in patient nail samples. J Invest Dermatol 2016;136:331-333.

Mignon B, Tabart J, Baldo A, Mathy A, Losson B, Vermout S. Immunization and dermatophytes. Curr Opin Infect Dis 2008;21:134-140.

Moyes D, Wilson D, Richardson J, Mogavero S, Tang S, Wernecke J, et al. Candidalysin is a fungal peptide toxin critical for mucosal infection. Nature 2016;532:64-85.

Nakamura Y, Kano R, Hasegawa A, Watanabe S. Interleukin-8 and tumor necrosis factor alpha production in human epidermal keratinocytes induced by *Trichophyton mentagrophytes*. Clin Diagn Lab Immunol 2002;9:935-937.

Netea M, Brown G, Kullberg B, Gow N. An integrated model of the recognition of *Candida albicans* by the innate immune system. Nat Rev Microbiol 2008;6:67-78.

Ohnemus U, Kohrmeyer K, Houdek P, Rohde H, Wladykowski E, Vidal-y-Sy S, et al. Regulation of epidermal tight-junction (TJ) during infection with exfoliative toxin-negative *Staphylococcus* strains. J Invest Dermatol 2008;128:906-916.

Perera T, Gregory D, Marshall D, Gow N. Contact-sensing by hyphae of dermatophytic and saprophytic fungi. J Med Vet Mycol 1997;35:289-293.

Peus D, Vasa R, Beyerle A, Meves A, Krautmacher C, Pittelkow M. UVB activates ERK1/2 and p38 signaling pathways via reactive oxygen species in cultures keratinocytes. J Invest Dermatol 1999;112,751-756.

Piérard G, Piérard-Franchimont C, Quatresooz P. Fungal thigmotropism in onychomycosis and in a clear hydrogel pad model. Dermatology 2007;215:107-113.

Proksch E, Brandner J, Jensen J. The skin: an indispensable barrier. Exp Dermatol 2008;17:1063-1072.

Sakulkoo W, Osés-Ruiz M, Garcia E, Soanes D, Littlejohn G, Hacker C, et al. A single fungal MAP kinase controls plant cell-to-cell invasion by the rice blast fungus. Science 2018;359:1399-1403.

Seebacher C, Bouchara J-P, Mignon B. Updates on the epidemiology of dermatophyte infections. Mycopathologia 2008;166:335-352.

Shiraki Y, Ishibashi Y, Hiruma M, Nishikawa A, Ikeda S. Cytokine secretion profiles of human keratinocytes during *Trichophyton tonsurans* and *Arthroderma benhamiae* infections. J Med Microbiol 2006;55:1175-1185.

Smijs T, Mulder A, Pavel S, Onderwater J, Koerten H, Bouwstra J. Morphological changes of the dermatophyte *Trichophyton rubrum* after photodynamic treatment: a scanning electron microscopy study. Med Mycol 2008;46:315-325.

Squeo R, Beer R, Silvers D, Weitzman I, Grossman M. Invasive *Trichophyton rubrum* resembling blastomycosis infection in the immunocompromised host. J Am Acad Dermatol 1998;39:379-380.

Tabart J, Baldo A, Vermout S, Nusgens B, Lapiere C, Losson B, et al. Reconstructed interfollicular feline epidermis as a model for *Microsporum canis* dermatophytosis.J Med Microbiol 2007;56:971-975.

Tani K, Adachi M, Nakamura Y, Kano R, Makimura K, Hasegawa A, et al. The effect of dermatophytes on cytokine production by human keratinocytes. Arch Dermatol Res 2007;299:381-387.

Tran VDT, De Coi N, Feuermann M, Schmid-Siegert E, Bagut ET, Mignon B, et al. RNA sequencing-based genome reannotation of the dermatophyte *Arthroderma benhamiae* and characterization of its secretome and whole gene expression profile during infection. mSystems 2016;1:00036-16.

Tomoyuki I, Kazushi A, Takashi M. Quantification of dermatophyte viability for evaluation of antifungal effect by quantitative PCR. Mycopathologia 2014;177:241-249.

Van Smeden J, Bouwstra J. *Stratum corneum* lipids: their role for the skin barrier function in healthy subjects and atopic dermatitis patients. Curr Probl Dermatol 2016;49:8-26.

Vermout S, Tabart J, Baldo A, Mathy A, Losson B, Mignon B. Pathogenesis of dermatophytosis. Mycopathologia 2008;166:267-275.

Weitzman I, Summerbell R. The dermatophytes. Clin Microbiol Rev 1995;8:240-259.

Zarubin T, Han J. Activation and signaling of the p38 MAP kinase pathway. Cell Res 2005;15:11-18.

Zhan P, Liu W. The changing face of dsermatophytic infections worlwide. Mycopathologia 2017;182:77-86.

Zhang Z, Wang B, Wu S, Wen Y, Wang, X, Song X, et al. PD169316, a specific p38 inhibitor, shows antiviral activity against Enterovirus71. Virology 2017;508:150-158.

# FIGURE LEGENDS

**Figure 1. Fungal hyphae invade *stratum corneum* (SC) in RHE by progressing through intercellular space. (a)** PAS staining with α-amylase treatment of histological sections prepared from RHE infected by *T. rubrum* arthroconidia for one (D0 + 1d), two (D0 + 2d), three (D0 + 3d) or four (D0 + 4d) days. Scale bar: 50 µm. **(b-c)** SC colonization and invasion of RHE by *T. rubrum* arthroconidia respectively assessed **(b)** by scanning electron microscopy performed one day after infection (D0 + 1d) or **(c)** by transmission electron microscopy performed four days after infection (D0 + 4d). Yellow dotted lines indicate limits between SC and *stratum granulosum*; scale bars: 5 µm.

**Figure 2. Infection of RHE by *T. rubrum* simultaneously induces epidermal barrier alterations and keratinocyte responses. (a)** Trans-epithelial electrical resistance and **(b)** permeability to Lucifer Yellow (LY) fluorescent dye of infected RHE compared with control and PBS-treated RHE (n=6; mean±SD; ●p<0.05 ●●●p<0.001; ANOVA1). **(c)** Fluorescence analysis of LY dye penetration in RHE and **(d)** localization of biotin by fluorescent-labelling after inside-out permeability assay on the fourth day following infection. Inset: negative control incubated without biotin; dotted lines show the filter; scale bars: 50 µm. **(e)** Expression and **(f)** release of pro-inflammatory cytokine (IL-8) and AMP (hBD2, hBD3) by keratinocytes of infected RHE compared with control and PBS-treated RHE and RHE exposed to heat-killed (HK) arthroconidia, respectively assessed by RT-qPCR and ELISA in culture media (n=3; mean±IC95 or mean±SD respectively for RT-qPCR or ELISA results; \*p<0.05 \*\*p<0.01 \*\*\*p<0.001 compared with D0; ANOVA1).

**Figure 3. p38 MAPK is constitutively phosphorylated in RHE and its activity can be inhibited by PD169316. (a)** Levels of p38 MAPK and phosphorylated p38 MAPK investigated by Western blotting, using antibodies specific for p38 MAPK or phosphorylated p38 MAPK, after protein extraction from RHE, during *T. rubrum* infection (0 to 4 days). **(b)** Levels of p38 MAPK and phosphorylated p38 MAPK, as well as levels of HSP27 and phosphorylated HSP27 assessed by Western blotting, using specific antibodies, on protein extracts from RHE previously treated or not for 24h with PD169316 (15 µM), a p38 MAPK specific inhibitor, and then exposed to H2O2 (1 mM) for 20 or 60 minutes. The detection of RPL13a protein was used as loading control.

**Figure 4. PD169316 hampers development of *T. rubrum* infection on RHE and prevents barrier alterations as well as keratinocyte responses.** RHE were cultured in presence or not of PD169316 (15 µM) and infected or not with *T. rubrum* arthroconidia for four days (D0 + 4d) before barrier analysis and investigation of keratinocyte responses. **(a)** Trans-epithelial electrical resistance and **(b)** permeability to Lucifer Yellow (LY) fluorescent dye in four studied conditions (n=3; mean±SD; \*p<0.05 \*\*p<0.01 \*\*\*p<0.001; ANOVA1). **(c)** Analysis by fluorescent microscopy of LY dye penetration illustrated for the four conditions. Dotted lines localize the filter; scale bars: 50 µm. **(d)** Same conditions observed byPAS staining with α-amylase pretreatment. Scale bars: 20 µm. **(e)** Relative mRNA expression of IL-8, hBD2 and hBD3 determined by RT-qPCR analysis of RNA extracts from RHE in same conditions (n=3; mean±IC95; \*p<0.05 \*\*p<0.01 \*\*\*p<0.001; ANOVA1).

**Figure 5. PD169316 impairs dermatophytes growth in culture on Sabouraud agar.** Dermatophytes arthroconidia were seeded on Sabouraud agar containing PD169316 (15 µM) or not (Control) and incubated for seven days at 27°C. **(a)** Culture dishes illustrating colonies (scale bar: 1 cm) andcomparison of *T. rubrum* growth determined by percentage of colony-forming units (CFU) versus Control (n=3; mean±SD; \*\*\*p<0.001; *t*-Student). **(b)** Scanning electron microscopy observation of *T. rubrum* colonies. Lower panels present at higher magnification hyphae in colony centers; scale bars: 100 µm. **(c)** PAS staining revealing infection by *T. rubrum* arthroconidia of lyophilized RHE and inhibition by PD169316. Scale bars: 20 µm. **(d)** and **(e)** illustrate growth percentage determined by CFU counting (n=3; mean±SD; \*p<0.05 \*\*p<0.01; *t*-Student) and macroscopic aspect of colonies (scale bar: 1 cm) obtained with*Trichophyton interdigitale*and *Trichophyton benhamiae*.

**Figure 6. Among five well-known p38 MAPK inhibitors, PD169316 is the only one that alters dermatophytes growth.** *T. rubrum* arthroconidia were seeded on Sabouraud agar containing 15 µM of different p38 MAPK specific inhibitors and incubated for seven days at 27°C. **(a)** Growth percentage of *T. rubrum* evaluated by colony-forming units (CFU) counting (n=3; mean±SD; \*\*\*p<0.001; *t*-Student) and **(b)** by microscopic observations of colonies in the presence of the inhibitors. Scale bars: 1 mm.