Experimental Models of Dermatophytosis

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Abstract

Dermatophytosis is a superficial fungal infection of the keratinized structures of the host. Since the last decade, this mycosis became an important health concern due to an increasing prevalence and to the limited number and efficacy of available treatments. Several experimental models have then been developed in order to improve knowledge about this infection and to design new therapeutic strategies. This chapter presents the variety of dermatophytosis experimental models and their contribution in the understanding of mechanisms used by dermatophytes to adhere and to invade the host tissue. Their support to study the establishment of effective antifungal defenses by the host is also summarized. The usefulness of these models for testing the efficacy of antifungal compounds is finally discussed.

Keywords

Dermatophytosis · *Trichophyton rubrum* · *Microsporum canis* · Experimental models · Skin equivalents · Reconstructed human epidermis · Adhesion · Invasion · Host responses · Antifungal efficacy

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Abbreviations

AMP	Antimicrobial peptides
CFU	Colony-forming unit
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IFNγ	Interferon-gamma
IL	Interleukin
Lap	Leucine aminopeptidase
Mep	Metalloprotease
NET	Neutrophil extracellular trap
PAS	Periodic acid-Schiff
PMN	Polymorphonuclear neutrophils
RFE	Reconstructed feline epidermis
RHE	Reconstructed human epidermis
Sub	Subtilisin protease
TGF	Transforming growth factor
TLR	Toll-like receptors
TNFα	Tumor necrosis factor alpha

1 Introduction

Dermatophytosis is the most common fungal disease in the world with a current prevalence estimated between 20% and 25% of the general population [1–4], but reaching more than 50% among some populations at risk such as diabetic patients or sport practitioners [5, 6]. This infection is caused by specific filamentous and keratinolytic fungi named dermatophytes. Several species are identified and classified into three groups according to their natural host or environment [7]:

- Geophilic species, for example *Nannizzia gypsea* and *Nannizzia fulva* (previously known as *Microsporum gypseum* and *Microsporum fulvum*, respectively), generally feed on keratinized wastes found in the environment and frequently remain nonpathogenic.
- Zoophilic species preferentially infect specific animal hosts but can also infect other animal species including humans. For example, the preferred hosts for *Microsporum canis* and *Trichophyton benhamiae* (previously known as *Arthroderma benhamiae*) are, respectively, cats and rodents.
- Anthropophilic species, as for example *Trichophyton rubrum* and *Trichophyton interdigitale*, exclusively infect humans.

Among all dermatophyte species able to cause infections in humans, *T. rubrum* is responsible for a vast majority of dermatophytosis in humans: indeed, depending on the geographical areas, between 50% and 90% of cases are due to *T. rubrum* [2, 8].

In immunocompetent patients, dermatophytosis affects the keratinized structures of the host, namely hairs, nails, and epidermis, resulting in superficial lesions with erythema, dryness, and desquamation as clinical symptoms [9, 10]. The severity of lesions partly depends on the species involved, zoophilic and geophilic dermatophytes usually inducing more severe inflammatory responses in human hosts in comparison with anthropophilic species that are well adapted to humans as a consequence of more efficient mechanisms for immunoevasion [11–13]. Anyway, lesions resulting from dermatophytosis cause pain and discomfort and their anesthetic appearance is clearly responsible for individual shame and low self-esteem in concerned patients, along with reduced social interactions and quality of life [14–16].

Despite their overwhelming prevalence, dermatophytosis has been yet poorly studied. Thus, many questions regarding the mechanisms deployed by dermatophytes to invade host tissues remain to be addressed. Similarly, other questions concerning the establishment of an adequate immune response in infected host require further investigation. Moreover, while antifungal agents currently available are effective against dermatophytes, these drugs have several limitations such as extended duration of the treatment, associated toxicity, especially encountered when oral administration is needed, and finally emergence of resistant strains [17].

During the last decades, several in vivo, in vitro, and ex vivo experimental models of dermatophytosis have been developed in order to answer outstanding questions. This chapter aims to describe various models useful for studying dermatophytosis, with a special focus on skin equivalent models, to depict their current contribution in the understanding of the multiple steps involved in host infection by dermatophytes, as well as to present their potential use in performing efficacy testing of antifungal compounds.

2 Experimental Models of Dermatophytosis

The increase in dermatophytosis prevalence observed since the last decade has gradually raised the interest of the scientific community for the study of this infection. This has led to the development and diversification of experimental models of dermatophytosis over the last few years.

2.1 Aleurioconidia or Arthroconidia as Infective Elements Initiating Dermatophytosis

Spores are quiescent fungal unicellular particles characterized by high mechanical resistance and low metabolic activity. They represent the initial stage of fungal development, able to reactivate, germinate, and produce new mycelium when environmental conditions are favorable [18]. Spores are physiologically produced by fungi to ensure survival in adverse conditions, as well as dispersion in the

environment of individuals which can adhere to host tissue before starting a new infectious process. Dermatophytes are able to produce two kinds of spores: aleurioconidia arising terminally or laterally from the hyphae (microconidia corresponding to one-celled aleurioconidia and macroconidia to several successive cells detaching together at the end of hyphae), and arthroconidia resulting from fragmentation of hyphae. Transmission and scanning electron microscopy analysis performed on aleurioconidia and arthroconidia adhering over corneocytes in suspension revealed that the cell wall of arthroconidia is thicker than that of aleurioconidia, and that germination of arthroconidia occurs faster after adhesion [19]. Moreover, arthroconidia appear more resistant to certain antifungal drugs (e.g., fluconazole, griseofulvin, itraconazole) than microconidia [20]. In addition to these intrinsic differences, aleurioconidia, although they are extensively produced by dermatophytes cultured in vitro, have never been observed in vivo on lesions [21]. Conversely, arthroconidia are efficiently produced in vivo by dermatophytes.

In order to create adequate models for dermatophytosis, the use of arthroconidia as infective elements seems therefore more appropriate. In practice, arthroconidia can be produced in vitro following the procedure described by Tabart et al. [22]. Practically, after approximately 2-week growth on nutrient-rich agar, the fungal mass is recovered by scrapping, and cultured for additional 2–3 weeks in conducive conditions that combine a nutrient-poor culture medium and a 12% CO_2 atmosphere. Unicellular elements, corresponding to arthroconidia, are finally isolated after agitation and filtration of the fungal material. However, aleurioconidia are used in most studies (e.g., [12, 23–26]), because of the ease and speed of their production in vitro: only 2-week culture on nutrient-rich agar before agitation and filtration steps. To date, only a few studies have been performed using arthroconidia (e.g., [19, 27–31]).

2.2 In Vitro, Ex Vivo, and In Vivo Models of Dermatophytosis

In the past, simple models of dermatophytosis were developed to describe the early steps of the infection, for instance, dermatophytes adhering to human corneocytes, either isolated after skin scrapping or in suspension, or invading sheets of cornified layer collected by the tape-stripping method [19, 30, 31]. More recently, models using nail or hair fragments infected by dermatophytes allowed the investigation of the mechanisms used by these fungi to degrade keratin [23] or permitted to characterize the expression of virulence factors by dermatophytes [24]. Although such models are suitable to monitor dermatophyte adhesion, growth, and, to a lesser extent, invasion, the absence of living keratinocytes impedes any study about the host tissue responses. Complementarily, in vitro models of keratinocytes [12, 26, 32, 33], polymorphonuclear neutrophils (PMN) [28], or macrophages [34] cultured in the presence of dermatophytes were designed to overcome those limitations. In vitro models allow the evaluation of expression and release of cytokines and antimicrobial peptides (AMP), together with the expression of toll-like receptors (TLR) or of co-stimulatory molecules by those cell types. However, even keratinocytes cultured

as monolayers cannot be used to accurately model the epidermal adhesion and invasion processes followed by dermatophytes since they lack keratinized material.

Indeed, an ideal model to study dermatophytosis should allow simultaneous analysis of the infection steps (i.e., adhesion, germination, and tissue invasion) used by dermatophytes on one hand, and analysis of the host responses that trigger the recruitment and activation of the antifungal immune defenses on the other. In addition, such a relevant model might provide an appropriate tool to perform efficacy and toxicity assays of antifungal compounds. Ex vivo models of skin explants and in vivo animal models easily fulfill these requirements and appear therefore as promising solutions. To date, skin explants from several animal species (e.g., cats, Guinea pigs, mice) as well as from human beings have already been used to study adhesion and invasion by dermatophytes [35–39] or to characterize their expression of potential virulence factors [24]. Nonetheless, the use of skin explants, especially from humans, is quite restricted by limited availability, poor standardization of samples (for instance, regarding hairiness or body area), and large variability between the donors.

Animal models, mainly based on Guinea pigs or mice experimentally infected by dermatophytes, have been used to study steps of infection (e.g., [35, 40, 41]) or to analyze the establishment of host immune responses (e.g., [25, 29, 42]). Several animal models used to study dermatophytosis have been recently reviewed in details by Cambier et al. [43]. Nevertheless, an accurate model of dermatophytosis should mimic infection by a specific dermatophyte on tissue from an adapted host. Thereby, studying the infection of animal tissue by typical anthropophilic dermatophytes is particularly irrelevant. Indeed, there is no report of natural infection of nonhuman epidermal tissue by anthropophilic species and experimental trials devoted to infect animal tissue by the same species remain complicated because anthropophilic dermatophytes are poorly adapted to adhere and invade nonhuman tissues. Since the anthropophilic *T. rubrum* species is the most common dermatophyte involved in human infections [2, 8], the availability of an accurate model of infection for this dermatophyte species is required. Despite the technical limitations mentioned, models of *T. rubrum* dermatophytosis using mouse [44, 45], Guinea pig [46–48], or rat [49, 50] have nonetheless been designed. Interestingly, repeated applications of spores, previous abrasive treatments, or injection of corticosteroids prior to and after the infection are clearly required in order to obtain significant lesions. Anyway, even if they are of a considerable interest to gain knowledge about activation of the immune system in the host organism in response to dermatophyte infection, one must keep in mind that critical differences between nonhuman and human epidermis might influence data interpretation. Furthermore, the animal models have obvious ethical concerns. Therefore, there is a strong case for the development of alternative models.

2.3 Skin Equivalents to Create Dermatophytosis Models

Human skin equivalents obtained in culture, including reconstructed epidermis alone or epidermis reconstructed on a dermal equivalent generally made of collagen lattice with fibroblasts, are currently the closest in vitro models that mimic cutaneous human tissues. In such models, human keratinocytes cultured at the air–liquid interface, in an environment containing appropriate growth factors and elevated Ca^{++} concentration, undergo a complete program of differentiation that creates a stratified reconstructed tissue covered by a keratinized layer, quite similar to the human epidermis [51]. Infection models developed on skin equivalents allow studies of interactions between host tissue and pathogens, i.e., analysis of the infection process, identification of responses triggered in keratinocytes, and characterization of potential alterations induced in the tissue function. Notably, studies about cutaneous infections by the yeast *Candida albicans* [52], *Staphylococcus aureus* bacteria [53], or helminths [54] have already proven the usefulness of skin equivalents to investigate the mechanisms underlying infection.

Several reports already described infection of skin equivalents by dermatophytes. In 1995, a model involving infection by *Trichophyton mentagrophytes* arthroconidia of reconstructed human epidermis (RHE) has demonstrated for the first time the ability of dermatophytes to adhere onto reconstructed tissues and to invade their cell layers [55]. Later on, a model of infection by *M. canis* arthroconidia on reconstructed feline epidermis (RFE) was created to allow investigation of the adhesion mechanisms used by this dermatophyte on its natural preferred host [22]. More recently, two models of dermatophytosis based on commercially reconstructed skin tissue EpiDerm (MatTek) [26] or EpiSkin® [56] were reported. The first model was explored to evaluate the release of cytokines by keratinocytes during the infection process by several dermatophyte species, including the anthropophilic *T. rubrum*. Characterization of the signaling pathways simultaneously involved was also undertaken [26]. By mean of morphological analysis, the second model of dermatophytosis on EpiSkin® illustrated the different steps of the infection process by *T. rubrum* [56]. Although both models brought interesting insight and information about dermatophytosis and its pathogenesis, they used aleurioconidia as infective elements. Since aleurioconidia are only produced by dermatophytes cultured in vitro and have never been observed in vivo [21], those models cannot be considered fully representative of the in vivo conditions of infection.

Our team designed a model of dermatophytosis using RHE grown on polycarbonate filter and then infected by *T. rubrum* arthroconidia [27]. Practically, infection of RHE is initiated by topical application of *T. rubrum* arthroconidia suspended in phosphate-buffered saline to reach a final density of 1700 arthroconidia per cm^2. After 4 h of exposure, washes are performed in order to eliminate non-adherent arthroconidia from the apical surface of RHE and to expose them again to the air–liquid interface. Infected RHE are then cultured during four additional days. Morphological analysis of infected RHE revealed that arthroconidia were able to rapidly adhere to the surface of corneocytes and to produce hyphae that progressively invaded the cornified layer (Fig. 1a). When infected RHE were maintained in culture

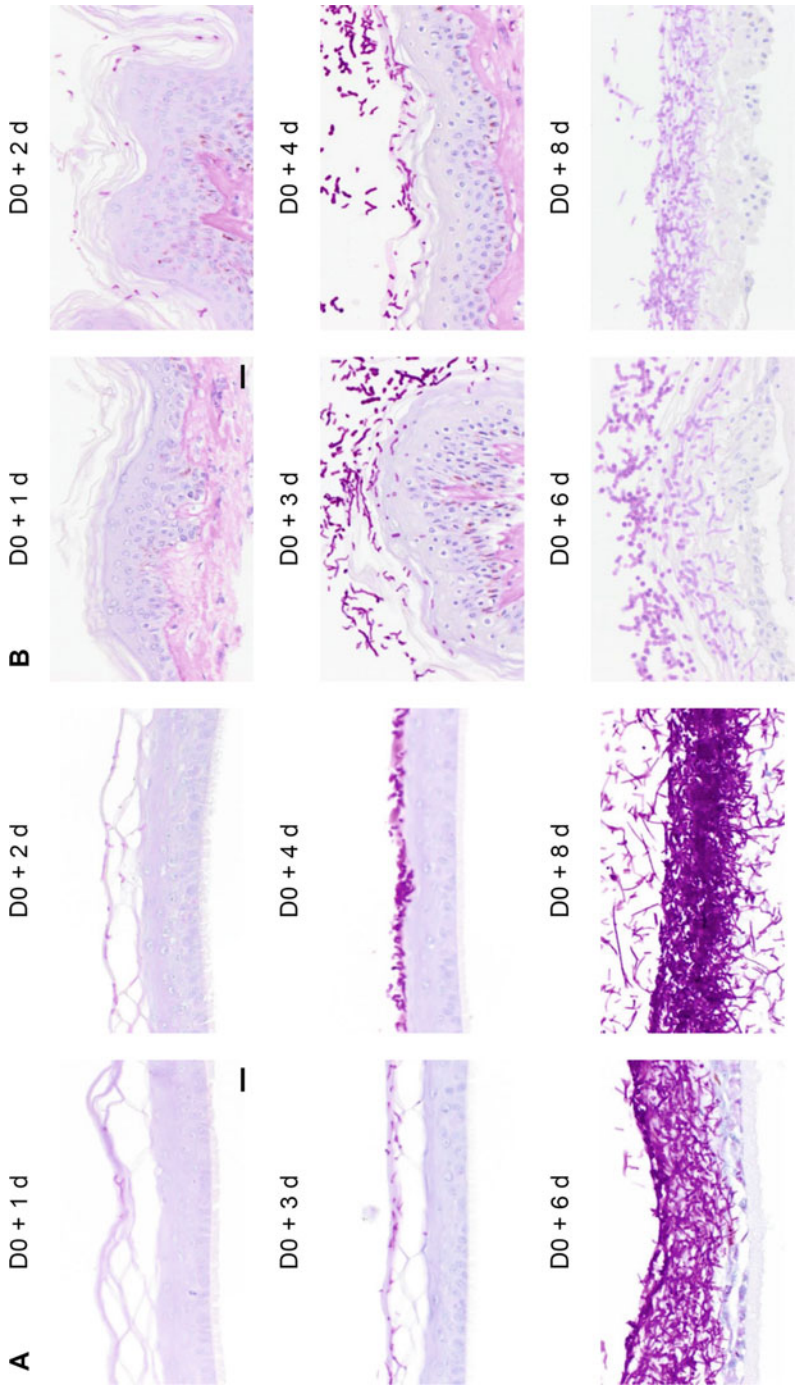

Fig. 1 In vitro infection of reconstructed human epidermis or of human skin explants by *Trichophyton rubrum* dermatophytes. Periodic acid-Schiff (PAS) staining with hemalun counterstaining of histological sections performed on infected (**a**) reconstructed human epidermis (RHE) or on (**b**) human skin explants, chemically fixed and processed for histology, one (D0 + 1 day), two (D0 + 2 days), three (D0 + 3 days), four (D0 + 4 days), six (D0 + 6 days), or eight (D0 + 8 days) days after exposure to *T. rubrum* arthroconidia. Histological sections of RHE were pretreated with α-amylase before the PAS staining. Normal human skin explants were obtained from abdominal plastic surgery after informed consent of patient (Drs. B. Bienfait and J.S. Blairvacq, Clinique St. Luc, Namur-Bouge, Belgium). Scale bars = 20 μm

for longer periods, hyphae progressed deeper between layers of the epidermis until entire invasion of the RHE, resulting in its total disorganization. This excessive invasion is for sure not representative of in vivo lesions since the progression of fungal elements is usually restricted to the cornified layer in naturally infected normal human skin [10]. This difference can likely be explained by the absence of immune cells in RHE-based models. Therefore, culture of infected RHE is systematically interrupted at latest 4 days after exposure to arthroconidia in order to keep the model representative of in vivo lesions. In addition, using the protocol developed for RHE infection, we demonstrated that infection by *T. rubrum* arthroconidia happens similarly on human skin explant (Fig. 1b). This model based on infection of RHE was explored to describe the progressive steps of infection [27], as well as to identify the cellular responses of host keratinocytes and the alterations of the barrier in the infected RHE [57, 58].

The aforementioned models of dermatophytosis on skin equivalent have proven their usefulness in the evaluation of infectious processes and of human keratinocytes responses, as well as in the identification of signaling pathways triggered during these responses. In addition, such models are valuable to test efficacy of antifungal compounds [27, 55, 59].

2.4 Complementary Models for the Study of Experimental Dermatophytosis

Every model described earlier allows advances in the study of dermatophytosis, but each of them is also more appropriate for the evaluation of certain aspects of the problem (Table 1). Thus, choosing a model depends essentially on the question addressed.

Adhesion is the initial step of infection during which fungal cells adhere to host substrate (i.e., keratinized structures). Ex vivo models of corneocytes in suspension and cornified layer sheets, or nail and hair fragments remain easy and adequate for the study of adhesion processes without any particular interference. Adhesion can also be studied using skin explants, skin equivalents, or animal models, but activation of host living cells (e.g., through production of AMP or recruitment of other immune components) and desquamation may influence the adhesion process. Invasion of the host tissue and consequences onto the epidermal barrier function can be evaluated in a similar manner using skin explants, skin equivalents, or animal models.

About the understanding of host tissue responses, through investigation of expression and release of cytokines, AMP, or other factors, in vitro, ex vivo, and in vivo models provide different and complementary information. In vitro models of cells cultured in suspension, as monolayers or inside RHE, allow monitoring the activation of specific cell types (e.g., keratinocytes, PMN, macrophages) independently of the others. Conversely, evaluation of the tissue reaction as a whole, including the activation of keratinocytes and of immune cells, either innate or adaptive, requires the use of in vivo animal models. For their part, ex vivo models

Table 1 Use of different experimental models to study specific aspects of the pathogenesis of dermatophytosis

	Adhesion	Invasion and effect on the epidermal barrier	Activation of the immune system	Activation of individual cell type	Antifungal efficacy
Ex vivo models					
Corneocytes, cornified layer sheets, nail and hair fragments	+				±
Skin explant	+	+	±	±	+
In vitro models					
Culture of keratinocytes, PMN, or macrophages				+	
Skin equivalents reconstructed in culture	+	+		+	+
In vivo models					
Animal models	+	+	+		+

using skin explants can be used to study the local responses given by either keratinocytes or cells of the innate immunity located in the epidermis and dermis of the explant.

Finally, testing efficacy of antifungal compounds is performed using ex vivo model of skin explant, in vitro model of skin equivalent, or in vivo animal model. Indeed, these three models cover the analysis of complete infection processes by dermatophytes and allow to evaluate the efficacy of antifungal compounds, whatever their mode of action. To a lesser extent, ex vivo models using corneocytes in suspension, cornified layer sheets, or nail and hair fragments, can be used to monitor the efficacy of compounds targeting the adhesion processes deployed by dermatophytes.

3 Experimental Models to Study Infectious Processes During Dermatophytosis

Dermatophytes infect host tissues through three successive steps: adhesion, germination, and invasion (Fig. 2a). In reaction to tissue invasion by dermatophytes, host cells become activated, most likely in order to initiate antifungal defenses and counteract the infection. Mechanisms involved during the infectious process as well as cellular responses triggered in host tissue can be both investigated by mean of experimental models of dermatophytosis, as mentioned above and depicted in details hereunder.

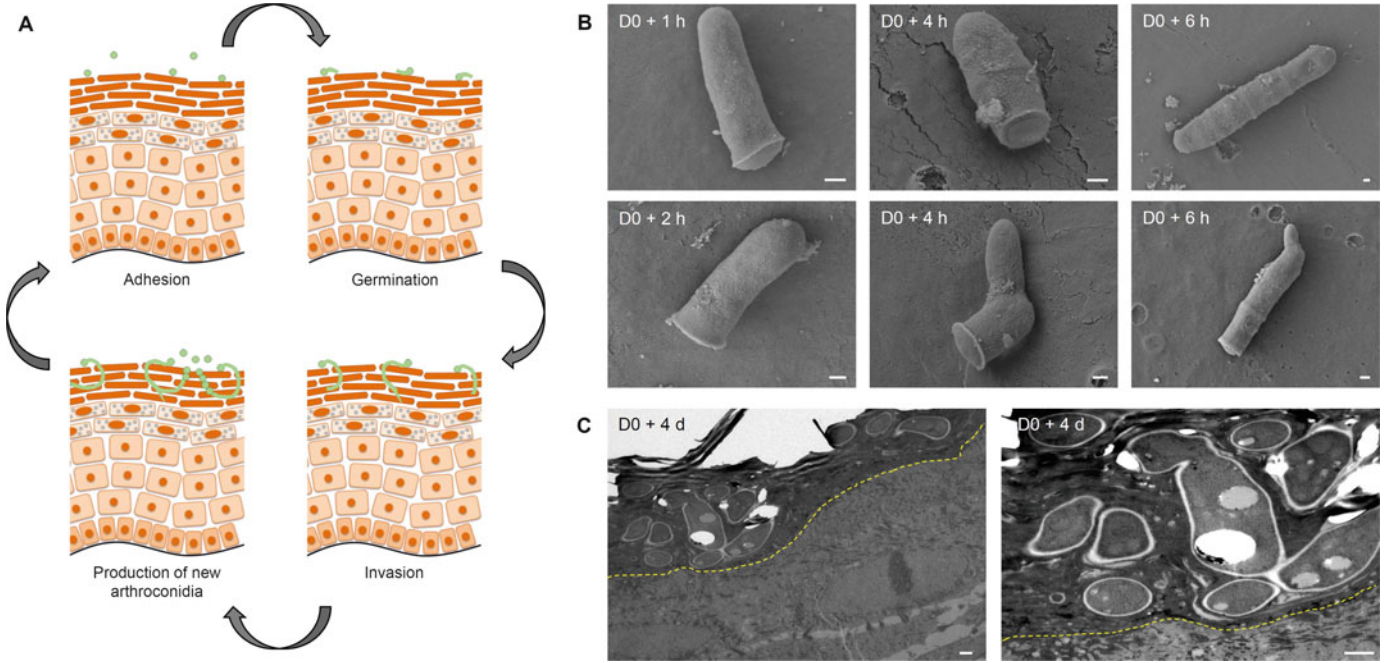

Fig. 2 Dermatophytes infect epidermis through four steps. (**a**) Schematic representation of the different steps observed during infection of epidermis by dermatophytes: adhesion of arthroconidia to tissue surface, germination of arthroconidia to become elongating hyphae, invasion of the cornified layer by hyphae, and finally production of new arthroconidia by hyphal fragmentation. (**b**) Adhesion and germination of *Trichophyton rubrum* arthroconidia over the surface of reconstructed human epidermis (RHE) observed by scanning electron microscopy one (D0 + 1 h), two (D0 + 2 h), four (D0 + 4 h), or six (D0 + 6 h) hours after exposure of the tissue to arthroconidia. Scale bars = 1 μm. (**c**) Invasion through the cornified layer of RHE by hyphae observed by transmission electron microscopy 4 days after infection (D0 + 4 days) by *T. rubrum* arthroconidia. Yellow dotted lines indicate the limits between cornified and granular layers. Scale bars = 1 μm

3.1 Adhesion to the Host Tissue

Adhesion is the first step of tissue infection by dermatophytes and involves close contact between arthroconidia and the surface of the host epidermis. Notably, analysis by electron microscopy have revealed that arthroconidia or microconidia from *T. mentagrophytes* and *T. interdigitale*, inoculated on human skin explants, produce fibrils that connect them to the epidermal surface [30, 38, 39].

3.1.1 Adhesion Is an Early Process in the Infection Development

Several studies performed on different experimental models of dermatophytosis have characterized the adhesion kinetics of dermatophytes to the host tissue. By light microscopy, Zurita and Hay [19] observed arthroconidia from three different *Trichophyton* species (i.e., *T. rubrum*, *T. interdigitale*, and *T. quinckeanum*) adhering to corneocytes in suspension. Their observations revealed that adhesion occurs as soon as after 2 h of contact, reaching a maximum after 4 h. Moreover, those authors report increased adhesion of *T. interdigitale* and *T. rubrum* to corneocytes from plantar skin, whereas the adhesion to corneocytes isolated from forearm skin is weaker, suggesting that the strength of dermatophyte adhesion varies upon body location. Accordingly, arthroconidia from *T. interdigitale* or *T. mentagrophytes* adhere to cornified layer sheets as soon as 1 h after tissue exposure and their adhesion increasingly occurred during the first 6 h of contact [30]. Other studies using microscopy further showed that adhesion of *T. mentagrophytes* on ex vivo models based on human nail fragments [60] or on human skin explants [38] started, respectively, 6 or 12 h after infection. Finally, adhesion of *M. canis* arthroconidia to the surface of RFE can be observed under a fluorescent light microscope after labeling arthroconidia with Calcofluor White. In this case, adhesion was shown starting within 2 h and increased up to 6 h after infection [61].

More recently, adhesion assays based on colony-forming units (CFU) counting have been developed. In one method, arthroconidia adhering to skin explants from human or other animal species were recovered by scrapping and were seeded over Sabouraud agar for a few days at 27 °C in order to assess the number of CFU which were adherent [35, 36]. This kind of assay demonstrated strong adhesion of *M. canis* arthroconidia over skin explants after 4 h of exposure. On the other hand, a second method developed using an in vitro model of dermatophytosis on RHE, rather assessed the number of non-adherent arthroconidia in a defined number laid over the epidermal tissue [27]. Practically, non-adherent arthroconidia were recovered by several washes of the tissue surface before being seeded over Sabouraud agar and counting of CFU as above. Knowing the initial number of arthroconidia entering in contact with the RHE, the number of adherent arthroconidia was determined by simple calculation. By using this method, it was shown that arthroconidia of *T. rubrum* start to adhere to RHE as soon as 1 h after infection and that *T. rubrum* adhesion increases for the first 24 h of exposure.

Altogether, these data suggest that adhesion is an early process in infection development and that it increases over time.

3.1.2 Surface Molecules and Secreted Proteases Are Required for Adhesion

Currently, the precise mechanisms used by dermatophytes to adhere to the host tissues are still incompletely understood. However, pieces of information have been obtained by the mean of experimental models of dermatophytosis. Interestingly, adhesion seems to rely on complex processes that simultaneously involve molecules expressed at the surface of arthroconidia and secreted proteases (reviewed by Baldo et al. [62]).

In such context, the study of interactions between *T. mentagrophytes* or *T. rubrum* with mutant Chinese ovary epithelial cells expressing various terminal carbohydrates on their cell surface revealed that mannose and galactose-binding proteins are present on the surface of microconidia [63, 64]. Accordingly, Bitencourt et al. [65] observed that *T. rubrum* aleurioconidia express a gene encoding an adhesin-like protein when they are cocultured with human keratinocytes. As other fungal adhesins [66–68], this adhesin-like protein contains a central domain with a tandem repeat sequence whose length and number of repeats likely influence adhesion ability of the different dermatophyte species or strains.

In addition, dermatophytes express a huge number of proteases, including subtilisin proteases (Sub), metalloproteases (Mep), and leucine aminopeptidases (Lap), which seem all implied in adhesion and invasion processes [62, 69]. Since the proteases Sub1, Sub3, Sub4, Lap1, Lap2, and Mep4 are highly expressed by dermatophytes when they are cultured in a protein-rich medium [40, 41, 65, 70, 71], they were long ago considered as virulence factors. Recent studies though have shown that proteases expressed by dermatophytes differ during in vivo infection: for instance, Sub6 is the main protease expressed by *T. benhamiae* during experimental infection on Guinea pig [40, 41], and by *T. rubrum* inside onychomycosis lesions naturally induced in humans [72]. Besides, even if Sub3 is not the main protease expressed in vivo, Sub3 was detected by immunohistochemistry in the skin of Guinea pig experimentally infected with *M. canis* [73]. Such a difference between in vitro and in vivo studies underlines the importance of suitable experimental models.

Proteases of the subtilisin family, especially Sub3, have been shown to have a major role in dermatophyte adhesion. Indeed, adhesion of *M. canis* arthroconidia to RFE surface is reduced by the chymostatin inhibitor which targets serine-proteases including subtilisins, and by specific antibody against Sub3, although in a lesser extent [61]. In addition, the adhesion of a *M. canis* strain invalidated for Sub3 by RNA silencing [74] to cutaneous explants from humans or other animals is decreased by comparison with a control strain of *M. canis* expressing Sub3 [35, 36]. Using an experimental model of dermatophytosis on RHE [27], we confirmed the involvement of subtilisin proteases in the adhesion of *T. rubrum* to the epidermal surface (Fig. 3). First, the inhibiting activity of chymostatin on that of Sub3 was verified, either in the absence and in the presence of the RHE, thanks to an assay based on an artificial substrate for this protease (*N*-succinyl-Ala-Ala-Pro-Phe-P-nitroanilide Sigma cat. no. S7388) (Fig. 3a). In addition, the viability of RHE was controlled when exposed to chymostatin (Fig. 3b). Infection of RHE was then

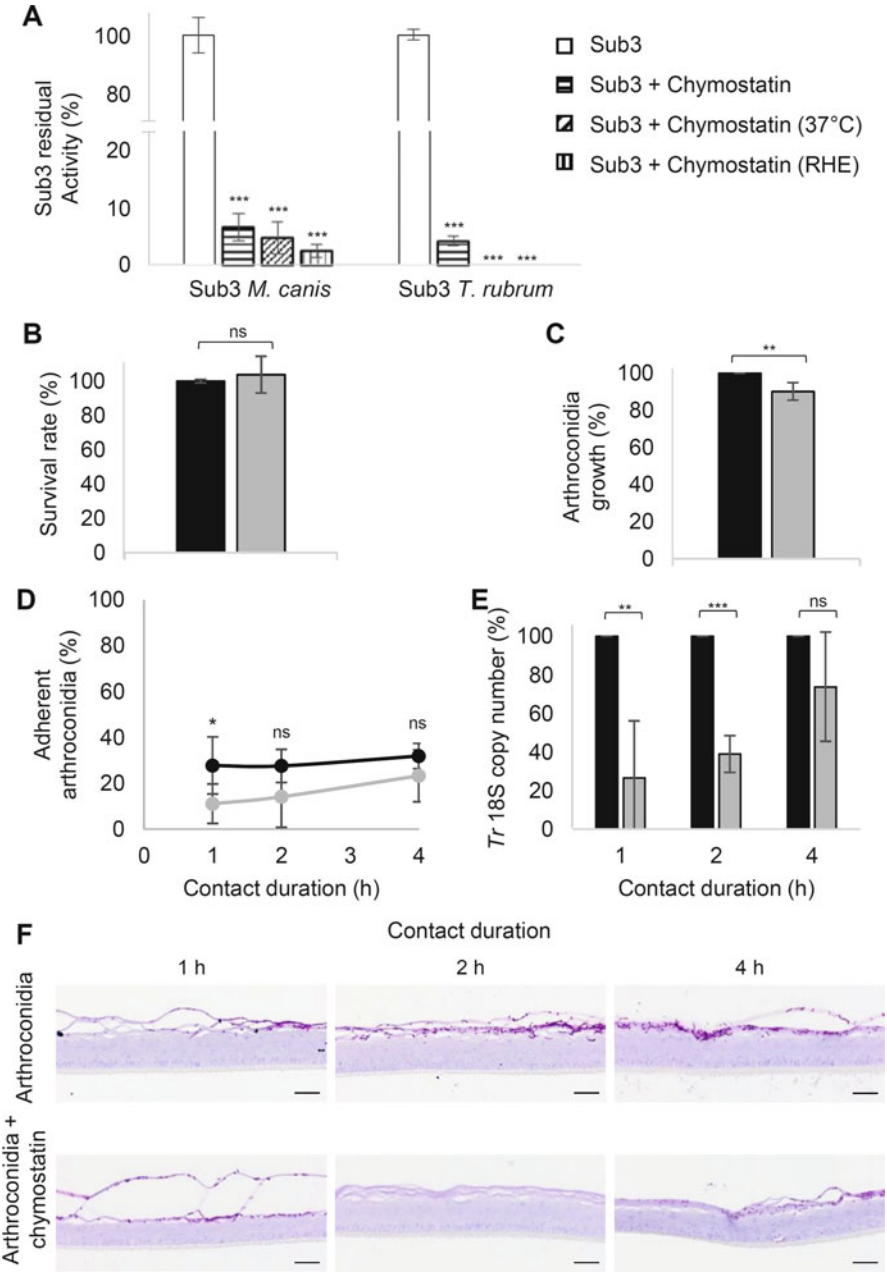

Fig. 3 Serine proteases are involved in the adhesion process of arthroconidia to host epidermis. (**a**) Residual activity of recombinant subtilisin-3 (Sub3) serine proteases from *Trichophyton rubrum* or *Microsporum canis* alone (white), in the presence of 100 μM chymostatin either under optimal conditions (horizontal hatchings), or after 4 h incubation at 37 °C (diagonal hatchings) or after 4 h incubation in topical application on reconstructed human epidermis (RHE) at 37 °C (vertical hatchings) ($n = 3 \pm$ SD; $^{***}p < 0.001$ in comparison to the activity of Sub3 alone; ANOVA1).

performed by topical exposure to *T. rubrum* arthroconidia, in the presence or not of chymostatin (100 μM), followed by washes of their surface in order to eliminate non-adherent arthroconidia after 1, 2, or 4 h of infection. Non-adherent arthroconidia recovered by washing were numbered as described above after seeding on Sabouraud agar, taking into account the effect of chymostatin on the growth of arthroconidia (Fig. 3c), in order to calculate the percentage of adherent arthroconidia (Fig. 3d). Adhesion is slightly reduced by the presence of chymostatin, especially when contact duration was as short as 1 h. This decrease in adhesion resulted, 4 days after infection, in a decrease in the number of fungi detected on the epidermal surface, as assessed by PCR quantification of the copy number of *T. rubrum* ribosomal DNA 18S (Fig. 3e) and by histological analysis after periodic acid-Schiff (PAS)-staining of infected RHE (Fig. 3f). Therefore, proteases of the subtilisin family appear clearly involved in the adhesion of dermatophytes to the host tissue, even if mechanisms promoting adhesion are still unclear.

3.2 Germination and Invasion of the Host Tissue

After adherence to host tissue, arthroconidia initiate germination. Once arthroconidia adhere to the host tissue, they are able to perceive that this environment is favorable for their growth and they initiate the germination step during which they may swell before producing germ tubes that will elongate to become hyphae [75]. Germination is initiated when arthroconidia produce a germ tube with a length at least equivalent to the one of the initial arthroconidia. In ex vivo models based on corneocytes in suspension [19] or on sheets of cornified layer [31], the germination of arthroconidia, respectively, from *T. interdigitale* and *T. mentagrophytes*, was observed after 4 h of incubation. Simultaneously though, aleurioconidia of *T. interdigitale* adherent to suspended corneocytes do not exhibit germination yet [19]. In accordance with a delayed germination of aleurioconidia, *T. mentagrophytes* microconidia inoculated on human skin explant seemed to start germination after 24 h [38], while germination of *T. rubrum* conidia was observed later, 2 days after infection on human skin

Fig. 3 (continued) (**b**) Survival rate of RHE after 4 h incubation in the presence of chymostatin (gray) compared with control untreated RHE (black) ($n = 3 \pm$ SD; $^{ns}p \geq 0.05$; *t* Student). (**c**) Growth of arthroconidia on Sabouraud agar in untreated conditions (black) or in the presence of chymostatin (gray) ($n = 4 \pm$ SD; $^{**}p < 0.01$; *t* Student). (**d**) Adhesion of arthroconidia to RHE in the presence (gray) or not (black) of chymostatin expressed as percent of the number of arthroconidia initially applied on the epidermal surface, depending on contact duration with RHE ($n = 3 \pm$ SD; $^{*}p < 0.05$, $^{ns}p \geq 0.05$; *t* Student). (**e**) Quantification of 18S rDNA gene copy number of *T. rubrum* by qPCR after total DNA extraction from infected RHE performed 4 days after infection by arthroconidia in the presence (gray) or not (black) of chymostatin, depending on the duration of contact with RHE ($n = 3 \pm$ SD; $^{**}p < 0.01$, $^{***}p < 0.001$, $^{ns}p \geq 0.05$; *t* Student). (**f**) Periodic acid-Schiff (PAS) staining with α-amylase pretreatment and hemalun counterstaining of histological sections prepared from RHE 4 days after their infection by *T. rubrum* arthroconidia, after 1, 2, or 4 h of exposure in the presence or not of chymostatin. Scale bars = 50 μm

equivalent [56]. By scanning electron microscopy performed on infected RHE (Fig. 2b), one can observe *T. rubrum* arthroconidia adhering to the tissue surface as early as after 1 h of contact. Later on, arthroconidia that become slightly swollen and germinate can be seen after 4 h. Elongating septate hyphae formed from germinated arthroconidia are observed after 6 h and become able to penetrate the host tissue during the invasion step.

During invasion, hyphae progress through the host tissue while continuing to elongate. Initial microscopic observations suggested that dermatophytes invade the host tissue by progressing through intercellular spaces without causing extensive damages to the cells. Indeed, 72 h after infection, *T. mentagrophytes* arthroconidia were observed invading sheets of cornified layer [31] or nail fragments [60] by progressing into intercellular spaces. In accordance, 3 days after infection, hyphae from *T. mentagrophytes* were found penetrating human skin explants between cells of the cornified layer and separating them [38]. In the dermatophytosis model on RHE developed by our team [27], we also observed using transmission electron microscopy analysis of infected tissues that hyphae are present in intercellular spaces between corneocytes 4 days after exposure to *T. rubrum* arthroconidia (Fig. 2c). These results suggest that dermatophytes are able to degrade intercellular junctions (e.g., corneodesmosomes or tight junctions) and/or the extracellular lipid matrix. In addition, these observations also suggest that hyphal elongation is orientated according to the physical and topographical features of the substrate in order to facilitate its invasion, a phenomenon known as "thigmotropism" [76]. This ability of dermatophytes to orientate their growth was also described by Perera et al. [77] who observed the growth of *Epidermophyton floccosum*, *M. canis*, and *T. mentagrophytes* on artificial membranes.

In addition to the above-described properties, dermatophytes secrete several proteases that exhibit specificity toward hard keratin substrates [69], rendering these fungi able to invade host tissue directly through corneocytes. Accordingly, scanning electron microscopy revealed penetration of corneocytes by *T. mentagrophytes* hyphae, resulting in damage to the corneocyte surface 21 h after the infection of cornified layer sheets [31]. Moreover, Jensen et al. [78] observed hyphae inside the corneocytes on human skin biopsy from *Tinea corporis* lesion. By infecting nail or hair fragments with various mutants of *T. benhamiae*, the critical role of the enzyme cysteine dioxygenase Cdo1 and of the sulfite efflux pump SSu1 in the degradation of keratinized structures by dermatophytes could be demonstrated [23]. Actually, thanks to Cdo1 and Ssu1, sulfite are produced by dermatophytes from environmental cysteine and secreted. As a reducing agent, sulfite can then cleave keratin-stabilizing cysteine bonds, rendering keratin more accessible for its degradation by proteases. Growth of strains deficient for one of the two (Cdo1 or Ssu1) proteins was indeed impaired on hair or nail fragments. Besides, Sub3, which seems involved in adhesion processes, is not required for invasion of the host tissue by dermatophytes. Indeed, arthroconidia from a *M. canis* strain invalidated for Sub3 produced hyphae able to invade the epidermis of experimentally infected Guinea pigs when the adhesion deficiency is artificially compensated by poloxamer 407, increasing the remanence of arthroconidia at the infection site [35].

It therefore appears that dermatophytes invade the host tissue by progressing simultaneously between and through corneocytes. Anyway, invasion of the cornified layer leads to alterations of the epidermal barrier, as observed on skin biopsy collected from *Tinea corporis* lesions [78]. Alterations of the epidermal barrier in response to dermatophyte infection of the in vitro RHE model were characterized by measurement of transepithelial electrical resistance, by assessing the outside-in permeability, together with the inside-out permeability [58]. While the two former assays confirmed loss of integrity in the epidermal barrier 4 days after the infection of RHE, the latter assay indicated that this loss can be partly due to the disorganization of tight junctions.

Finally, it is important to note that the infection by dermatophytes in vivo remains superficially localized in the keratinized structures (i.e., cornified layer of the epidermis, nails, and hairs), their progression into living tissues being probably blocked by the host immune system [7, 10]. However, in dermatophytosis models based on skin equivalent [27, 56] or on skin explant [38], the invasion reaches deeper epidermal layers in a few days, leading to the disorganization of the host tissue. This can be explained by the total absence of immune cells in skin equivalents, whereas cells from the innate immunity are sometimes present in skin explants, even if adaptive immunity is nonetheless missing. Those observations highlight the importance to monitor the development of infection in experimental models in order to perform assays and measurements when invasion is similar to that observed in vivo during natural infection.

3.3 Host Responses Against Dermatophytosis

Host responses against infecting dermatophytes include both activation of local cells and recruitment and activation of immune cells. Additionally, some dermatophytes also deploy several mechanisms to evade or silence host immune response.

3.3.1 Recruitment of the Immune System Through Activation of Local Cells

Experimental animal models are necessary to study the entire recruitment and activation of the host immune system in response to dermatophytosis. Firstly, Green et al. [79] demonstrated the requirement of cell-mediated immunity to fight against dermatophytosis by observing that nude mice experimentally infected by *T. mentagrophytes* were unable to heal. In accordance, Calderon and Hay [80] showed that transfer of T cells from mice acutely infected by *T. quinckeanum* into naive mice resulted in their protection against infection by this fungus. Conversely, serum transfer did not confer any protection. Later, secretion of interferon-gamma (IFNγ) and recruitment of macrophages were detected in the skin of wild-type mice experimentally infected by *T. rubrum*, whereas IFNγ or interleukin (IL)-12 knockout mice presented higher fungal burdens and lower macrophage recruitment than wild-type animals [45]. Those observations suggested that the Th1 immune response,

implying IFN-γ production and macrophages activation, was the effective response allowing to control and resolve infection by dermatophytes [81, 82].

However, emerging data tend to show that the Th17 immune response could also be required for the control of dermatophyte infection [25, 29]. Indeed, using mouse models based on *T. quinckeanum* [83], *T. mentagrophytes* [84], or *T. benhamiae* [42], it was shown that PMN were recruited in large numbers to the infection site. Secretion of cytokines involved in the Th17 response (i.e., IL-6, IL-17A, IL-23, and transforming growth factor (TGF)-β) was also reported during experimental infection by *T. mentagrophytes* and *T. benhamiae* on mouse models [42, 84]. The involvement of the Th17 immune response in dermatophytosis clearance was finally clearly demonstrated in a recent study comparing *M. canis* infection on wild-type or IL-17RA or IL-17A/F-deficient mice: while it remains superficially localized in the cornified layer of wild-type mice, *M. canis* extensively colonizes the epidermis when the Th17 pathway is dysfunctional [25].

Interestingly, the recent report by Heinen et al. [29] reconciled all data since they observed that T cells isolated from the skin-draining lymph nodes of mice experimentally infected by *T. benhamiae* exhibit both Th1 and Th17 differentiation as assessed through cytokines production (IL-17A, IL-22, IFNγ) and transcription factors mRNA expression (retinoic acid receptor-related orphan receptor γt and T-box transcription factor). In addition, they showed that fungal clearance and clinical recovery are lower in IFNγ and IL-17A double-deficient mice than in IFNγ or IL-17A single-deficient mice, suggesting complementary roles of Th1 and Th17 immune responses.

On another hand, some studies performed on simpler models, such as cells cultured either in suspension or in monolayers, as well as skin equivalents reconstructed in culture, allowed to monitor the specific activation of various cell types in response to dermatophytosis. Keratinocytes are the first cells to encounter dermatophytes and to react to their presence by production of cytokines and AMP. Indeed, human keratinocytes cultured as monolayers, in the presence of different dermatophyte species, exhibit release of several pro-inflammatory cytokines such as IL-1α, IL-1β, IL-8, and tumor necrosis factor alpha (TNFα), growth factors like granulocyte-macrophage colony-stimulating factor (GM-CSF), and AMP including β-defensins-2 and -3 and protein S100A7 [12, 13, 26, 32, 33]. Similarly, mRNA expression and release of pro-inflammatory cytokines and AMP by keratinocytes embedded in skin equivalents are observed in such models exposed to infection by dermatophytes [26, 58]. Responses triggered in keratinocytes appear dependent on the dermatophyte species selected for epidermal infection, the release of cytokines being generally higher in the presence of zoophilic species, such as *T. benhamiae* or *T. mentagrophytes*, than in the presence of anthropophilic dermatophytes, i.e., *T. rubrum* or *Trichophyton tonsurans* [12, 13]. This reflects the high or low inflammatory levels observed in cutaneous lesions induced in vivo by the different species of dermatophytes and pledges again for the right model selection when studying specific dermatophytosis. Besides, the mRNA expression of TLR-2, -4, and -6 by keratinocytes cultured as monolayers was modulated during stimulation by *T. rubrum* [85].

By analysis of PMN maintained in culture, the release of IL-1β, IL-8, and TNFα and the production of neutrophil extracellular traps (NET) by this cell type were observed when PMN are incubated in the presence of *T. benhamiae* or *M. canis* [26, 86]. Overexpression of TLR-2 and -4 mRNA was further characterized in PMN exposed to *M. canis* [87]. In addition, release of TNFα and IL-1β by macrophages challenged by *T. rubrum* was measured on a simple culture model [34, 88]. Finally, IL-6, IL-8, IL-12, and TNFα were produced by dendritic cells upon contact with *T. benhamiae* or *M. canis* ([86]; Tabart, unpublished data).

Considering all these data, the antifungal response by the host tissue during epidermal dermatophytosis has been summarized in Fig. 4. Initially, keratinocytes detect the presence of dermatophytes through the recognition of specific fungal motifs by TLR, mainly TLR-2, -4, and -6. Activated keratinocytes start producing various pro-inflammatory cytokines, including IL-1α, IL-1β, IL-8, and TNFα, which become upon release responsible for the recruitment of PMN and macrophages. Simultaneously, dendritic cells also detect dermatophytes via TLR or dectin receptors and subsequently participate to inflammation by their own secretion of pro-inflammatory cytokines. Thereafter, dendritic cells mature to become antigen-presenting cells and migrate into the lymph nodes in order to induce the differentiation of Th1 and Th17 cells, respectively, through the production of IL-12 or IL-6. Activated T cells then migrate to the infection site and secrete specialized cytokines: IFNγ by Th1 cells, IL-17 and IL-22 by Th17 cells. IFNγ stimulates macrophages which then become able to produce pro-inflammatory cytokines and to phagocyte small fungal elements. In the same time, the production by keratinocytes of PAM, responsible for direct antimicrobial effects, and GM-CSF is induced by IL-17 and IL-22. Finally, PMN are activated by GM-CSF and participate to the clearance of dermatophytes by phagocytosis, production of NET, and secretion of pro-inflammatory cytokines.

3.3.2 Modulation of Host Immune Response by Dermatophytes

Certain dermatophytes might dispose of mechanisms to evade or silence the immune response, causing chronic and low inflammatory lesions. Particularly, the anthropophilic *T. rubrum* species exhibits inhibition of macrophage functions and an induced secretion of anti-inflammatory proteins by macrophages and keratinocytes. Indeed, exposure of cultured macrophages to *T. rubrum* resulted in down-modulation of the major histocompatibility complex class II, in reduction of expression of co-stimulatory molecules, and in a upregulated release of IL-10 [34]. Moreover, *T. rubrum* further induced expression and release by keratinocytes of the anti-inflammatory protein encoded by TNFα-stimulated gene 6 [58]. Altogether, properties of *T. rubrum* which favor its adaptation to human host may explain why this species induces lower inflammatory lesions than zoophilic or geophilic dermatophytes.

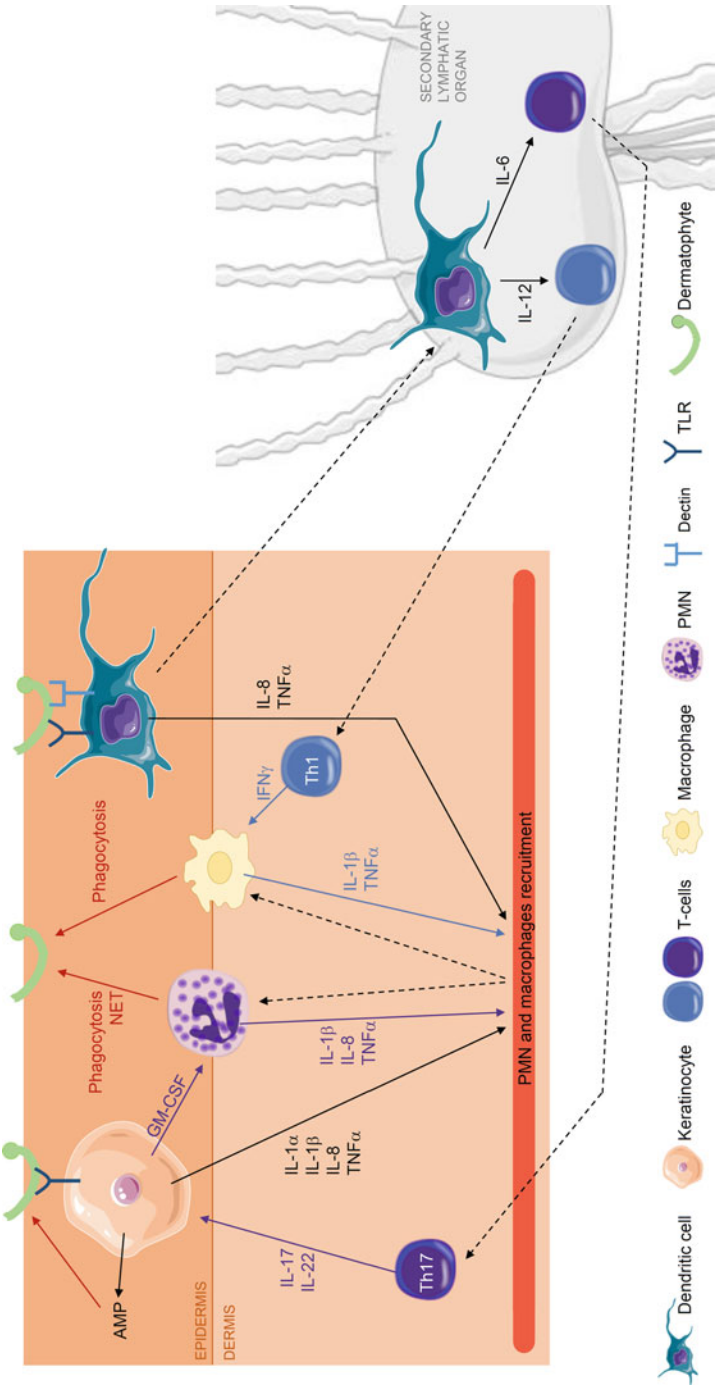

Fig. 4 Innate and adaptive immune responses are induced during dermatophytosis. Host keratinocytes and dendritic cells detect the presence of dermatophytes invading the epidermis through specific receptors, i.e., toll-like receptors (TLR) and dectin. They then produce and release antimicrobial peptides (AMP) and pro-inflammatory cytokines which initiate the recruitment of macrophages and polymorphonuclear neutrophils (PMN). Dendritic cells migrate to secondary lymphatic organs where they induce the differentiation, activation, and recruitment of Th1 and Th17 cells. Th1 cells, through the release of IFNγ, improve the recruitment and activation of macrophages. In the same time, IL-17 and IL-22, produced by Th17 cells, enhance the release of AMP and cytokines by keratinocytes and induce the release of GM-CSF activating PMN. Finally, macrophages and PMN, through phagocytosis and neutrophil extracellular traps (NET) formation, as well as AMP by their direct antimicrobial activities, are together responsible for the control and the eventual resolution of infection

4 Experimental Models to Evaluate Efficacy of Antifungal Compounds

Although skin lesions produced during dermatophytosis may heal spontaneously within a few months, treatment is necessary in most cases. Several antifungal agents effective against dermatophytes are currently available. The most frequently used molecules are azole derivatives or terbinafine, both acting by inhibition of ergosterol synthesis, an essential compound for the organization of normal membrane in fungal cells [89]. Antifungal molecules are used for topical application or systemic administration, depending on the extension, severity, and accessibility of the lesion [17, 90]. For instance, local administration is chosen to treat simple lesions of *Tinea corporis* or *Tinea pedis*, while systemic administration is often required for nail or hair lesions, as well as for severe or recalcitrant cutaneous lesions.

Despite the current availability of effective antifungal agents, the management of dermatophytosis must face several problems [17]. Firstly, the treatment of dermatophytosis may become long-lasting and thus expensive, leading too often to reduced compliance in patients who generally stop taking medication as soon as the clinical symptoms improve. Premature interruption of antifungal treatment contributes to the occurrence of an increasing number of cases of relapses and reinfections. Secondly, the systemic administration of some antifungal molecules is unfortunately accompanied by side effects on the central nervous system (e.g., headache, dizziness, concentration difficulties), on the gastrointestinal system (e.g., nausea, diarrhea, abdominal pain), and on the skin (e.g., erythema, rash, pruritus). In addition, toxicity for liver of systemic administration must be seriously considered [91]. Finally, treatments for dermatophytosis must cope with the emergence of drug resistance [92–94].

In view of problems linked to the existing treatments, but also because of an increasing incidence of dermatophytosis, the development of new antifungal compounds effective against dermatophytes becomes a real priority.

As explained above in this chapter, experimental models are essential to improve the current understanding of dermatophytosis pathogenesis, and are thus in good position to help in identifying new potential targets for antifungal strategies. In addition, experimental models allow an easy evaluation of the efficacy of antifungal compounds. Notably, animal models were considerably developed in the past for this purpose; for instance, terbinafine and itraconazole were both demonstrated efficient by the analysis of Guinea pigs experimentally infected by *T. mentagrophytes* or *M. canis* aleurioconidia [95, 96]. Since most antifungal agents are developed for human treatment, mainly targeting *T. rubrum* species, animal models of *T. rubrum* infection were also developed based on mouse [44] or Guinea pigs [46, 48], despite the difficulties in infecting animal with an anthropophilic dermatophyte (see Sect. 2.2).

Because the use of animal models is increasingly challenged, limited and even banned for ethical and political reasons, alternative methods are now considered. Thereby, ex vivo models of dermatophytes adhering to cornified layer sheet [97] or to corneocytes in suspension [19] were used to monitor the impact of photodynamic

therapy or of antifungal molecules (i.e., ketoconazole, itraconazole, and griseofulvin) on adherence between dermatophytes and host cells. Furthermore, skin equivalent models were proved to provide valid methods to evaluate antifungal efficacy by using reference antifungal compounds such as terbinafine [55] or miconazole [27, 59]. Using a model of dermatophytosis on RHE, our group lately identified PD169316, a well-known specific inhibitor of the human p38 mitogen-activated protein kinase, as a potential antifungal agent effective against dermatophytes [58]. Indeed, PD169316 exhibits some direct effect on growth of fungi and thereby interferes with the infection of RHE by *T. rubrum* arthroconidia. Finally, a model of infection based on silkworm was recently developed by injection of *T. mentagrophytes* aleurioconidia in this invertebrate, in order to evaluate antifungal agents against dermatophytosis [98].

Therefore, each model appears as useful experimental device to identify and evaluate the efficacy of new antifungal molecules while, except for sheets of cornified layer, assessing potential toxicity for the treated tissue. In addition, one must keep in mind that arthroconidia are the infective elements produced in vivo by dermatophytes [21] and are more resistant than microconidia to some antifungal compounds [20]. In consequence, experimental models using arthroconidia as infective elements (e.g., [27, 55, 59]) must be considered as more appropriate to perform efficacy assay of antifungal molecules.

5 Conclusion

This chapter summarized experimental models for the investigations of both the infection process deployed by dermatophytes and the development of the host immune response. A better understanding of the pathogenesis of dermatophytosis will allow the identification of potential therapeutic targets and the subsequent development of new antifungal compounds. Since each type of model, whether ex vivo, in vitro, or animal model, holds characteristics bringing intrinsic advantages and limitations that make each of them more or less appropriate for precise investigations, the choice of one or another model must always consider the purpose of the study. For example, while simple in vitro models of keratinocytes cultured as monolayers remain useful to study particular responses of this host cell type exposed to dermatophytes, they are certainly less appropriate to investigate the recruitment of host immune cells and the activation of immunity. Conversely, animal models are suitable to evaluate the entire host immune responses, but do not allow in-depth focus on the activation of individual cells. In conclusion, a complete understanding of dermatophytosis will still depend upon the deployment of complementary experimental models.

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