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Optimisation of a Murine Infection Model With *Trichophyton mentagrophytes* for Studying the Pathogenesis of Dermatophytosis

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Received: 18 September 2025 | **Revised:** 10 November 2025 | **Accepted:** 26 November 2025

Keywords: dermatophytoses | fungal virulence marker | genotyping | host cytokine | *Trichophyton mentagrophytes*

ABSTRACT

Background: Dermatophytes are the most common agents of superficial mycoses in humans and animals. In a model of *Trichophyton benhamiae* dermatophytosis in its natural host (guinea pig), the most overexpressed gene was subtilisin 6 (SUB6). Given the availability of powerful genetic and immunological tools in mice, murine models of dermatophytosis should be developed using strains that can mimic natural infections.

Objective: The aim of this study was to test a strain of *Trichophyton mentagrophytes* isolated from a rodent in a murine skin infection model, to characterise the expression of key host and fungal genes and investigate the role of SUB6 in virulence by mimicking a natural infection as closely as possible.

Results: A phylogenetic tree was generated to better discriminate the *T. mentagrophytes* strains isolated from animals. The *T. mentagrophytes* TIMM 2789 strain used in this study is genotype IV, specific to rodents. Infection induced symptoms and lesions, including hair follicle invasion, typical of acute superficial dermatophytosis. Early overexpression of genes encoding specific cytokines revealed the involvement of the Th1, Th2 and Th17 responses by the host, and the overexpression of the fungal *SIDC* gene underscores the importance of iron acquisition during infection. The use of deleted and complemented *SUB6* strains revealed that SUB6 does not appear to be necessary for fungal virulence, while *SUB5* overexpression suggests a compensatory mechanism.

Conclusion: This study demonstrates the crucial importance of carefully selecting the most appropriate dermatophyte strain for the animal species in the experimental model used.

Abbreviations: *ARP2*, naphthalene reductase gene; ATCC, American Type Culture Collection; BD2, β-defensin 2; CBS, Centraalbureau voor Schimmelcultures; cDNA, complementary DNA; CFU, colony-forming unit; *DEUT*, deuterolysin gene; *DPP IV*, deuterolysin IV gene; *IFNγ*, interferon-γ gene; IHEM, Institute of Hygiene and Epidemiology Mycology; *IL*, interleukin gene; *IRF5*, interferon regulatory factor 5 gene; ITS, internal transcribed spacer; mRNA, messenger RNA; NCBI, National Center for Biotechnology Information; PAS, periodic acid–Schiff; PBS, phosphate-buffered saline; PI, post-infection; RT-qPCR, reverse transcription–quantitative polymerase chain reaction; SGA, Sabouraud glucose agar; *SIDC*, nonribosomal siderophore peptide synthase gene; SNI, scarified but non-infected; SUB, subtilisin; *SUB*, subtilisin gene; *THAU*, thaumatin gene; TIMM, Teikyo Institute of Medical Mycology; TLR, Toll-like receptor; Tm, *Trichophyton mentagrophytes*; Tm-SUB6c, *T. mentagrophytes* TIMM 2789 with *SUB6* complementation; Tm-WT, wild-type *T. mentagrophytes* TIMM 2789; Tm-ΔKU80, *T. mentagrophytes* TIMM 2789 with *KU80* deletion; Tm-ΔSUB6, *T. mentagrophytes* TIMM 2789 with *SUB6* deletion; *TNFα*, tumour necrosis factor alpha gene; Treg, regulatory T cell; WT, wild-type.

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1 | Introduction

Dermatophytosis is one of the most common fungal infections worldwide, with an estimated prevalence of 20%–25% [1–3]. Most contributions on dermatophytes concern taxonomy, epidemiology and treatments. In-depth research on the pathophysiological mechanisms involved in dermatophytosis is much more limited compared with other important fungi such as *Candida* and *Aspergillus* species. Some research on the pathogenicity of dermatophytes has focused on the secretion of proteases, which facilitate tissue invasion. Dermatophytes can secrete keratinolytic enzymes such as metalloproteases and serine proteases of the subtilisin (SUB) family to break down keratin, a key component of skin and nails [4]. The keratinolytic SUB3 of *Microsporum canis* is involved in adhesion to the epidermis [5], and SUBs are generally considered to be virulence factors [6, 7]. In a model of *Trichophyton benhamiae*–infected guinea pigs, which are the natural hosts of this species of dermatophyte, among the SUB family members, *SUB6* showed the highest upregulation (it was the second most highly upregulated gene overall), followed by *SUB8* and *SUB10* [8].

One of the main challenges in researching virulence factors of dermatophytes, such as SUBs, and host defence mechanisms is the availability of reliable animal models. Guinea pigs have been the main animals used in in vivo models [9]. One advantage of guinea pigs is that they allow the use of certain species of dermatophytes, such as *T. benhamiae*, which naturally infects this host, enabling the study of pathophysiological mechanisms under conditions as close to a natural infection as possible. However, the limitations of the guinea pig model are the absence of inbreeding and the lack of immunological tools for this species. Furthermore, the operating costs and ethical issues, guinea pigs being often kept as pets, make it difficult to use this model [9]. Given these drawbacks, using a mouse model would be preferable to improve our understanding of dermatophytosis pathogenesis. The wide variety of mouse strains available, including those with genetic deficiencies for specific immunological components, as well as the abundance of genetic and immunological tools for this species, make mice particularly useful for research on host–pathogen interactions, including dermatophytosis [10–13]. With a mouse model, it would therefore be important to use a strain of dermatophyte isolated from mice or other similar rodent species to reproduce a natural infection as closely as possible.

The objective of this study was to test in mice a strain of *Trichophyton mentagrophytes* isolated from a rodent, as well as a mutant strain in which the gene encoding a presumed virulence factor, *SUB6*, had been deleted. After genotyping the selected *T. mentagrophytes* TIMM 2789 strain, originally isolated from a chinchilla, the different aspects of the host–pathogen relationship based on the expression of various key host and fungal genes were investigated. Through this approach, we highlighted the involvement of the Th1, Th2 and Th17 responses by the host and the importance of iron acquisition by the fungus during infection. The use of *SUB6*-deleted and complemented strains revealed that SUB6 does not appear to be necessary for fungal virulence, while *SUB5* overexpression suggests a compensatory mechanism.

2 | Materials and Methods

2.1 | Dermatophyte Strains and Genetic Manipulation

Wild-type (WT) *T. mentagrophytes* TIMM 2789 (American Type Culture Collection [ATCC] 28145; Centraalbureau voor Schimmelcultures [CBS] 646.73; Institute of Hygiene and Epidemiology Mycology [IHEM 3299])—denoted as Tm-WT in this paper—was originally isolated from chinchilla [14]. It was obtained from the Teikyo Institute of Medical Mycology (TIMM) culture collection. This strain has been widely employed in genetic research, particularly in studies aimed at developing essential molecular tools [15].

A *T. mentagrophytes* mutant with *KU80* deletion (Tm-ΔKU80) was produced from Tm-WT by a Japanese team [16]. Tm-ΔKU80 was used as the recipient strain for genetic manipulation to obtain a strain with *SUB6* deletion (Tm-ΔSUB6) as well as a strain with *SUB6* complementation (Tm-SUB6c) [17]. Figure S1 provides additional details about genetic transformation to generate Tm-ΔSUB6 and Tm-SUB6c.

2.2 | Sequencing and Phylogenetic Analyses

Genomic DNA was extracted from fresh dermatophyte cultures on Sabouraud glucose agar (SGA). The ribosomal internal transcribed spacer (ITS) region was amplified with the primers fwd 5′-GGTTGGTTTCTTTTCCT-3′ and rev 5′-AAGTAAAAGTCGTAACAAGG-3′. The ITS sequence was used for direct sequencing and compared with those available in the dermatophytes from the National Center for Biotechnology Information (NCBI) database.

Complete ITS sequences from a selection of *T. mentagrophytes* strains (Table S1) were aligned using ClustalW as implemented in the Geneious software (Figure S2). Trees were generated on partial ITS sequences of 570 sites using Tamura–Nei and neighbour-joining methods. The final tree results from evaluating candidate trees (bootstrap 1000 replicates). The *Trichophyton equinum* ITS sequence was used as an outgroup.

2.3 | Culture Conditions and Production of Fungal Inoculum for Infection

All *T. mentagrophytes* strains (Table S2) were grown on SGA at 30°C. Spore suspensions were produced by following a published method [18]. Briefly, after growth on SGA, dermatophyte sporulation was induced by incubation on potato dextrose agar at 30°C and 12% CO_2. The fungal material was harvested, filtered through Miracloth layers (Millipore, Overijse, Belgium) and unicellular spores were suspended in phosphate-buffered saline (PBS). The total concentration of spores in the resulting suspension was determined by counting colony-forming units (CFU) after incubation on SGA for 3 days at 30°C.

To produce germ tubes and mycelium mixture, 1×10^8 CFU were grown in Sabouraud glucose broth for 24 h at 30°C under

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agitation (110 rpm). The germ tubes and mycelium were harvested on Miracloth layers and washed twice with 20 mL of PBS.

2.4 | Murine Infection Model

All animal experiments were conducted in accordance with European regulations. The protocols were approved by the Walloon authorities and the Ethics Committee of the University of Liège (agreement number: 22-2479).

The mice were infected as previously described [19]. Briefly, epicutaneous inoculation was performed in two steps: (1) 100 mg of a mixture of germ tubes and mycelium was applied to 1 cm [2] of a scarified area on the occipital zone; then, (2) 100 μL of a sterile 5% Poloxamer 407 solution (Merck, Darmstadt, Germany) containing 1×10^8 CFU was added to the top.

2.5 | Clinical Score Analysis

After infections, the mice were monitored in double-blind conditions every 2 days by two independent examiners, who remained the same throughout the experiment, until the skin symptoms had completely disappeared. The examiners assigned a score based on the intensity of three clinical signs: erythema (rated from 1 to 4), scaling (rated from 1 to 4) and crusting (rated from 1 to 4). Then, a cumulative clinical score was derived from the addition of the three clinical signs (a maximum of 12). Skin biopsies were taken after Days 2 and 5 post-infection (PI) for histological analysis and total RNA extraction as previously described [19].

2.6 | Histological Analysis

Biopsies taken from infected mice were fixed in a 4% formaldehyde and histologically processed as previously described [19]. The samples were stained with periodic acid–Schiff (PAS) and counterstained with haematoxylin to highlight fungal elements.

2.7 | RNA Extraction and RT-qPCR

Total RNA was extracted from mouse skin biopsies and reverse transcribed into complementary DNA (cDNA) following the procedures previously optimised [19]. After qPCR using Takyon NO ROX SYBR Master Mix (Eurogentec, Seraing, Belgium) and specific primers (listed in Tables S3 and S4), the relative messenger RNA (mRNA) expression of murine and fungal genes was calculated using the $2^{-\Delta\Delta Ct}$ method [20]. For analyses of the host immune response, the mRNA expression levels were normalised relative to the transcript levels measured in skin samples from scarified but non-infected (SNI) mice. These samples were collected on Days 2 and 5 after scarification, which corresponded to the same time points analysed during infection. For fungal gene expression analyses, the mRNA expression levels were normalised relative to the transcript levels measured in the fungal inoculum (a mixture of complex mycelium and hyphae) used for infection (time point 0), which served as the reference control condition.

2.8 | Statistical Analysis

The G*Power3 software was used to calculate the number of mice required for a meaningful interpretation of the results. Using a one-sided proportion test with a risk of error (α) of 5% and power of 95%, three mice per replicate and study day were necessary for clinical score analysis, and two mice per replicate and study day were necessary for histological and molecular analysis of skin biopsies. All statistical analyses were performed using RStudio version 4.4.2. The Shapiro–Wilk test was used to determine whether the data followed a normal distribution. Then, the data were analysed with a two-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc test, with $p \leq 0.05$ considered to indicate a statistically significant difference.

3 | Results

3.1 | The *T. mentagrophytes* TIMM 2789 Strain Is Genotype IV

After DNA extraction and sequencing, the Tm-WT ITS sequence showed 100% identity and 98% query coverage with sequence AF170452 corresponding to *T. mentagrophytes* IHEM 10162 isolated from a chinchilla (Table S1).

Phylogenetic analysis revealed that Tm-WT belongs to genotype IV (Figure 1). All strains of genotype IV have been isolated from rodents and can be divided into three distinct subgroups, each corresponding to strains isolated from a major rodent clade: Hystricomorpha (IVa), Myomorpha (IVb) and Sciuromorpha (IVc). They all share the 115 substitution (C → T), while there are unique polymorphisms at position 2 (G → C) exclusively in strains isolated from mice (subgroup IVb), as well as at positions 188 (A → *gap) and 448 (G → A) in strains isolated from squirrels (subgroup IVc).

We observed two additional zoophilic groups based on host associations, with typus III comprising strains isolated from canine and feline hosts, and typus V comprising strains isolated from herbivorous hosts. The clustering of each ITS genotype based on phylogenetic analysis and association with hosts is summarised in Table 1.

3.2 | Pathophysiology of Infection With the Wild-Type *T. mentagrophytes* TIMM 2789 Strain in Mouse

We used our model of superficial dermatophytosis in mice to investigate the pathogenic mechanisms involved in infection by the *T. mentagrophytes* TIMM 2789 strain. The mice were infected with Tm-WT and clinically evaluated over time using a clinical score based on erythema, scaling and crusting (Figure 2A,B). While scaling and crusting developed strongly from Day 2 until Day 6 PI, then tended to decrease over time, erythema was partially masked by crusting until Day 6 PI and increased from that point onwards. The cumulative clinical score revealed a lesional peak on Day 6 PI, mainly characterised by obvious scaling and crusting. As previously reported for *T. benhamiae* [19], we found a significant difference between male

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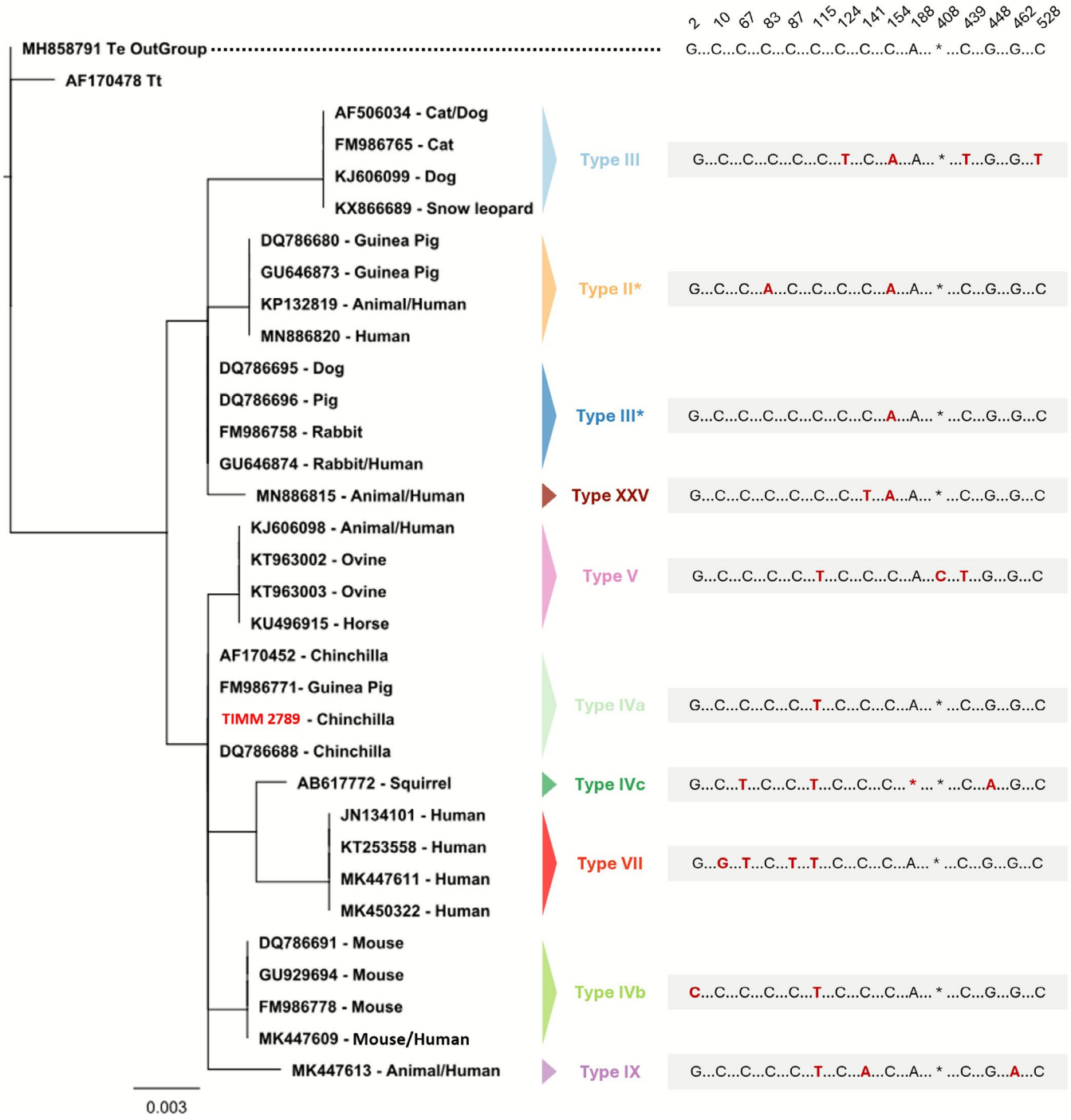

FIGURE 1 | Phylogenetic tree from *Trichophyton mentagrophytes* internal transcribed spacer (ITS) sequences and corresponding nucleotide mutations. An alignment of 33 partial *T. mentagrophytes* ITS sequences was performed using ClustalW as implemented in the Geneious software. The final tree is the result of evaluating candidate trees generated by the Tamura–Nei and neighbour-joining methods (1000 bootstrap replicates). The *Trichophyton equinum* (Te) ITS sequence was used as outgroup. Each sequence is identified by its GenBank accession number and listed in Table S1. The scale bar represents the number of nucleotide substitutions per site, indicating evolutionary distances among the sequences. For more details about multiple sequence alignment of the *T. mentagrophytes* ITS sequences, see Figure S2. Orange, typus II* strains isolated from guinea pig and human hosts; light blue, typus III strains isolated from canine and feline (carnivorous) hosts; dark blue, typus III*; green, typus IV strains isolated from rodent hosts (IVa, Hystricomorpha; IVb, Myomorpha; IVc, Sciuromorpha); pink, typus V strains isolated from herbivorous hosts; red, typus VII strains isolated from human hosts; purple, typus IX; brown, typus XXV. Tt, *Trichophyton terrestre. T. mentagrophytes* TIMM 2789 is highlighted in red in the tree. In the sequences, specific mutations are indicated in red and asterisks (*) indicate gaps.

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TABLE 1 | Classification of *Trichophyton mentagrophytes* internal transcribed spacer (ITS) genotypes among human and animal hosts.

ITS genotype	ITS sequence	Zoophilic or anthropophilic	Reservoir host	Classification	References
I	AF506033	Anthropophilic	Human	Human	[21]
II	AF506036	Anthropophilic	Human	Human	[21]
II*	**GU646873/ KP132819**	**Zoophilic**	Guinea pig, human	—	[21–23]
III	**AF506034**	**Zoophilic**	Cat, dog, snow leopard	Canidae/Felidae (carnivorous)	[22, 23]
III*	**GU646874/ FM986758**	**Zoophilic**	Dog, pig, rabbit	Mainly rabbit (Lagomorpha)	[22–24]
IVa	**AF170452**	**Zoophilic**	Chinchilla, guinea pig	Rodent (Hystricomorpha)	[22, 23]
IVb	**GU929694/ FM986778**	**Zoophilic**	Mouse, human	Rodent (Myomorpha)	[22, 23, 25, 26]
IVc	**AB617772**	**Zoophilic**	Squirrel	Rodent (Sciuromorpha)	[27]
V	**KU496915**	**Zoophilic**	Ovine, horse	Artiodactyla/ perissodactyla (herbivorous)	[28, 29]
VII	MK447611	Anthropophilic	Human	Human	[30, 31]
VIII	MH791420	Anthropophilic	Human	Human	[32–34]
IX	**MK447613**	**Zoophilic**	Animal, human	—	[32, 34]
XXV	**MN886815**	**Zoophilic**	Animal, human	—	[32, 34]

Note: Bold text is for zoophilic species.

and female mice regarding the erythema, the crusting and the cumulative scores (Figure 2B). Given the more severe nature of the lesions observed in males, we only used male mice for the subsequent experiments.

To assess tissue invasion by Tm-WT and the cellular inflammatory response in the host, we performed histological analysis on skin biopsies taken from Days 2 to 6 PI, when the cumulative clinical score was the highest (Figure 2C). On Day 2 PI, fungal spores were mainly observed, and crust formation had begun. Subsequently, we observed hyphae, which gradually invaded the growing crust, resulting in the formation of a rather dense fungal cluster on Day 5 PI. As fungal invasion of the tissues progressed, there was increasing infiltration of inflammatory cells in the dermis. It is striking that *T. mentagrophytes* exhibited the ability to invade hair shafts and follicles as early as Day 4 PI, a feature that has not been observed in experimental infections with *T. benhamiae* in mice [19]. In summary, Days 2 and 5 PI are key moments for *T. mentagrophytes*, corresponding respectively to the onset of epidermal invasion and the peak of acute infection (characterised by the maximal fungal load).

At the same time, the expression of several murine genes encoding different key players involved in the skin immune response was assessed by RT-qPCR on Days 2 and 5 PI after total RNA extraction (Figure 2D). All nine genes studied were strongly overexpressed in infected mice compared with SNI mice on Day 2 PI. More specifically, genes involved in the Th1 (*IFNγ*, *IL-12β* and *TNFα*), Th2 (*IL-5*), Th17 (*IL-1β, IL-6*) and regulatory T cell (Treg) (*IL-10*) differentiation pathways, as well as *BD2* which encodes the anti-microbial peptide β-defensin 2, were overexpressed during the early phase of infection. On Day 5 PI, only *IL-6* remained upregulated.

To determine whether Tm-WT expresses fungal markers during infection in our mouse model, we selected five genes based on previously published data [19] and evaluated their expression (Figure 2E). Naphthalene reductase (*ARP2*), deuterolysin (*DEUT*), and nonribosomal siderophore peptide synthase (*SIDC*) were strongly upregulated during infection compared with the control (hyphae cultured in liquid medium) on Days 2 and 5 PI. Thaumatin (*THAU*) was only slightly upregulated on Day 5 PI.

3.3 | The *T. mentagrophytes* SUB6 Null Mutant Induces Superficial Skin Inflammatory Lesions Similar to Those Induced by the Reference Control Strains in Mice

The *T. mentagrophytes KU80*-lacking mutant (Tm-ΔKU80), derived from the wild-type (Tm-WT) strain, was used as the recipient strain to generate a *SUB6*-deleted (Tm-ΔSUB6) strain and a *SUB6*-complemented (Tm-SUB6c) strain (Figure S1 and

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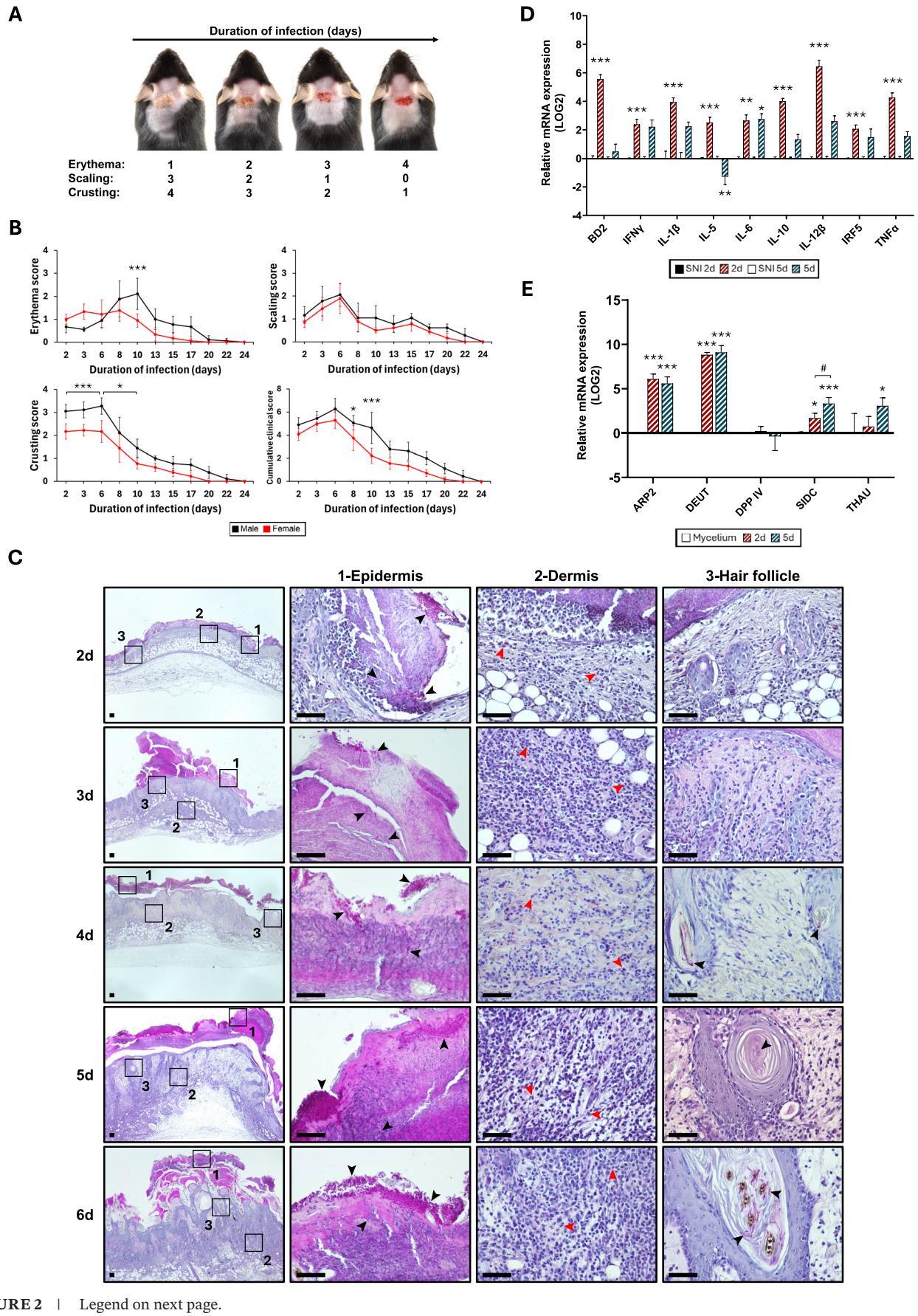

FIGURE 2 | Legend on next page.

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FIGURE 2 | Development of a mouse model of infection with wild-type *Trichophyton mentagrophytes* TIMM 2789 (Tm-WT): Clinical analysis, histological analysis, expression profile of genes associated with the host inflammatory response and fungal markers. Male and female mice were infected with *T. mentagrophytes* wild-type (Tm-WT). (A, B) Skin lesions developed by infected mice were monitored until complete recovery, with a clinical focus on the intensity of erythema, scaling and crusting. A cumulative clinical score was calculated based on these three clinical signs ($n = 9 \pm$ SD; ANOVA; *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$; asterisks show significant differences observed during infection between male and female mice). (C) Skin biopsies were collected from Days 2 to 6 post-infection (PI), when the cumulative clinical score was high, and then processed for histology and periodic acid–Schiff staining with haematoxylin counterstaining. For each time point, the first column shows a general view of the infected area, the second column shows the epidermal zones where the fungal invasion is contained, the third column shows the dermis and the inflammatory infiltrate, and the fourth column shows the hair follicles. Fungi are indicated by black arrowheads (➤) and neutrophils by red arrowheads (➤). The scale bar is 50 μm. (D, E) The relative messenger RNA (mRNA) expression of (D) genes encoding mouse major cytokines and an anti-microbial peptide (BD2) and (E) fungal markers was assessed by RT-qPCR after total RNA extraction. The mRNA expression was normalised using the level of the same transcript measured under control conditions (scarified but non-infected [SNI] mice for murine genes; or the fungal complex mixture [mycelium/hyphae] just before inoculation for fungal markers) ($n = 6 +$ SD; ANOVA2; */# $p < 0.05$, **/## $p < 0.01$ and ***/### $p < 0.001$; asterisks show significant differences compared with the fungal control condition; and black hash marks show significant differences between strains). Complete names and accession numbers of the studied genes are listed in Tables S3 and S4. 2d, Day 2; 3d, Day 3; 4d, Day 4; 5d, Day 5; 6d, Day 6.

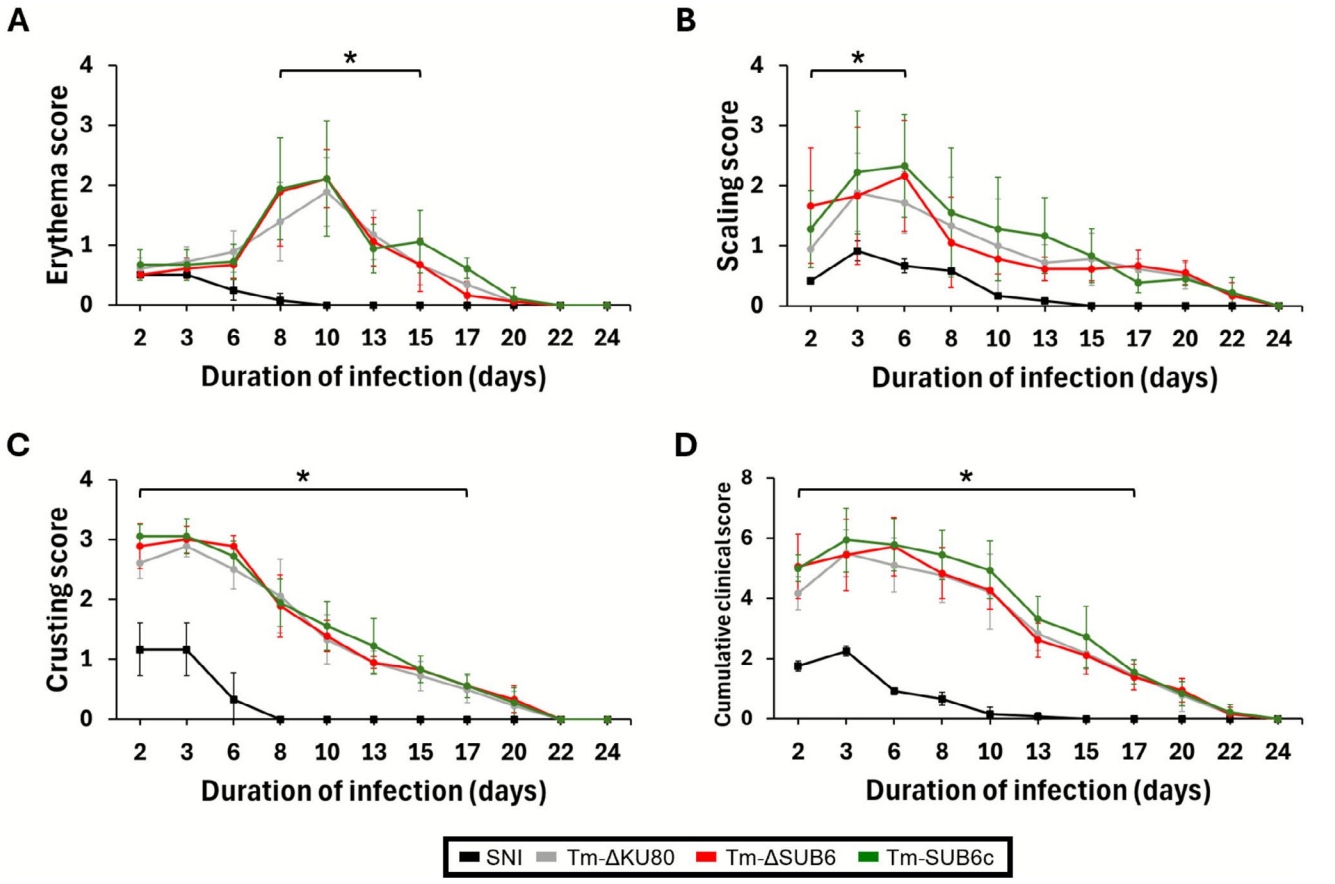

FIGURE 3 | Clinical scores of mice during infection by *T. mentagrophytes KU80*-lacking mutant (Tm-ΔKU80; recipient strain), *SUB6* deleted (Tm-ΔSUB6) or *SUB6* complemented (Tm-SUB6c) strains. The lesions that developed in the infected mice were monitored until complete recovery, with clinical emphasis on the intensity of (A) erythema, (B) scaling and (C) crusting. (D) A cumulative clinical score was calculated based on these three clinical signs ($n = 9 \pm$ SD; ANOVA2; *$p < 0.05$; asterisks show significant differences observed during infection between each of the three strains compared with scarified but non-infected [SNI] mice).

Table S2). Mice were infected with each of these strains; significant differences in the clinical score were observed between males and females for all strains (Figure S3). Furthermore, no significant difference was observed between the clinical scores measured during infection by Tm-WT and Tm-ΔKU80 strains (Figure S4). We also examined the expression of the fungal marker in the *KU80*-lacking mutant (Tm-ΔKU80). This strain showed gene expression that was comparable to that of Tm-WT (Figure S5). Mice infected with Tm-ΔKU80, Tm-ΔSUB6 or Tm-SUB6c strains exhibited similar clinical scores, suggesting that the absence of the *SUB6* gene does not impair the development of the infection (Figure 3).

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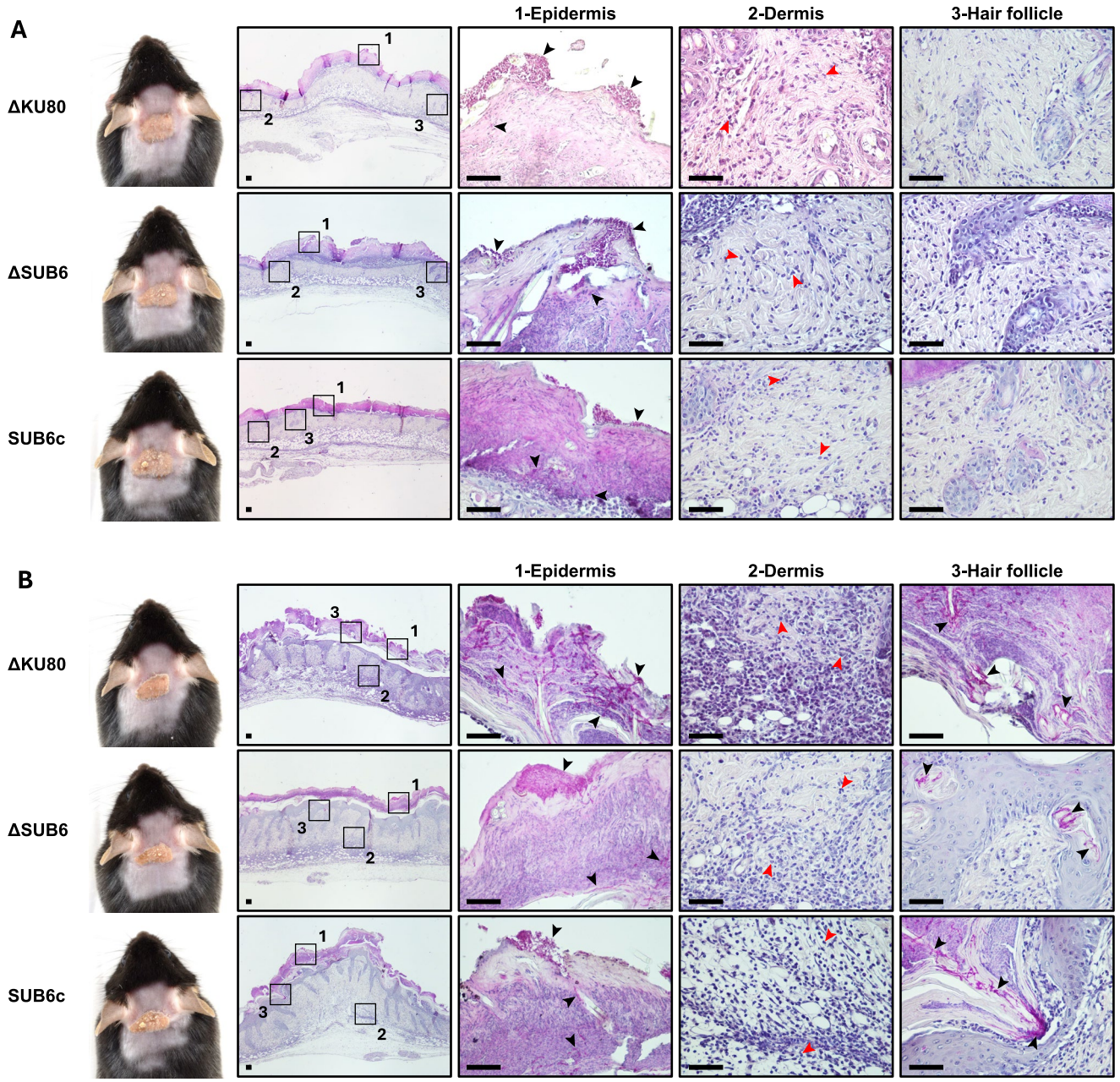

FIGURE 4 | Similar fungal invasion and inflammatory infiltrate between mice infected by the *SUB6*-deleted strain (Tm-ΔSUB6) and reference control strains. Mice were infected with *T. mentagrophytes KU80*-lacking mutant (Tm-ΔKU80; recipient strain), *SUB6* deleted (Tm-ΔSUB6) or *SUB6* complemented (Tm-SUB6c) strains. Skin biopsies were collected on (A) Day 2 (2d) and (B) Day 5 (5d) post-infection (PI) and then processed for histology and periodic acid-Schiff staining with haematoxylin counterstaining. For each time point, the first histological column shows a general view of the infected area, the second column shows the epidermal zones where the fungal invasion is contained, the third column shows the dermis holding the inflammatory infiltrate and the fourth column shows the hair follicles. Fungi are indicated by black arrowheads (➤) and neutrophils by red arrowheads (➤). The scale bar is 50 μm.

Histological observations corroborate the previous observation suggesting that the absence of *SUB6* does not alter the virulence of *T. mentagrophytes* in our model. On Days 2 and 5 PI, fungal invasion into keratinized tissues and infiltration of the dermis including perifollicular areas by mononuclear and polymorphonuclear cells did not differ between the mice infected with Tm-ΔSUB6 and Tm-ΔKU80 or Tm-SUB6c (Figure 4). In addition, Tm-ΔSUB6 demonstrated the ability to grow in hair follicles on Day 5 PI, as it is the case for the two other strains.

3.4 | The Overexpression of Genes That Encode Pro-Inflammatory Cytokines and β-Defensin 2 Is Similar in Mice Infected With the *SUB6*-Deleted Mutant and Reference Control Strains

The relative mRNA expression of pro-inflammatory cytokines and *BD2* was also examined during mice infection by Tm-ΔKU80, Tm-ΔSUB6, or Tm-SUB6c strains (Figure 5A,B). On Day 2 PI, *IFNγ*, *IL-1β*, *IL-6*, *IL-10*, *IL-12β*, *IRF5* and *TNFα* were significantly

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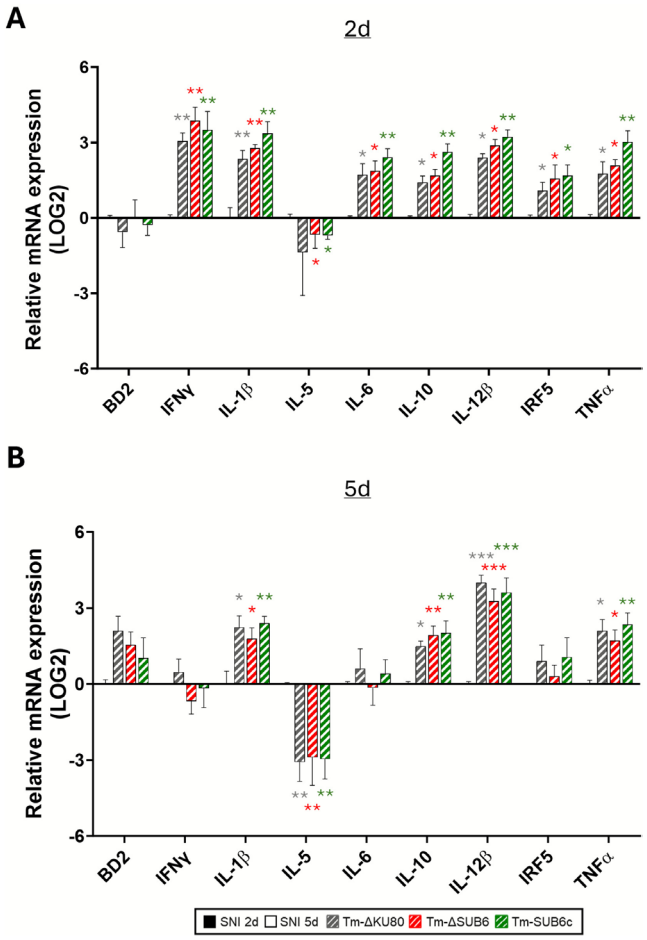

FIGURE 5 | Inflammatory responses of the host during infection in mice by the *SUB6*-deleted strain (Tm-ΔSUB6) and reference control strains. Mice were infected with the *T. mentagrophytes KU80*-lacking mutant (Tm-ΔKU80; recipient strain), *SUB6* deleted (Tm-ΔSUB6) or *SUB6* complemented (Tm-SUB6c) strains. The relative messenger RNA (mRNA) expression of murine pro-inflammatory cytokines and the anti-microbial peptide (BD2) was assessed by RT-qPCR after total RNA extraction from skin biopsies on (A) Day 2 (2d) and (B) Day 5 (5d) post-infection (PI). The mRNA expression was normalised using the level of the same transcript measured under control conditions (scarified but non-infected [SNI] mice) ($n = 6 + \text{SD}$; ANOVA2; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$; coloured asterisks show significant differences compared with the control condition for each strain). Complete names and accession numbers of studied genes are listed in Table S3.

overexpressed compared with the SNI mice, but with no significant difference between the different mutants. On Day 5 PI, only *IL-1β*, *IL-10*, *IL-12β* and *TNFα* remained overexpressed.

3.5 | The *SUB5* Gene of the Subtilisin Family Is Overexpressed by the *T. mentagrophytes* SUB6 Null Mutant in Mice

The potential compensatory or inhibitory mechanisms between SUBs during infection were studied. Specifically, we compared the mRNA expression of SUBs on Days 2 and 5 PI in mice infected with Tm-WT, Tm-ΔKU80, Tm-ΔSUB6, or Tm-SUB6c (Figure 6A,B). There was significant and strong overexpression of *SUB1*, *SUB3*, *SUB5*, *SUB6*, *SUB8* and *SUB10* for all strains (except for *SUB6* in Tm-ΔSUB6) during infection compared with the control (hyphae cultured in liquid medium). Strikingly, *SUB5* was significantly more overexpressed in Tm-ΔSUB6 on Day 5 PI than in the other three strains.

4 | Discussion

An optimised mouse model of dermatophytosis was used to study the pathophysiology of infection by *T. mentagrophytes* TIMM 2789 strain, which originates from chinchilla. We highlighted the involvement of the Th1, Th2 and Th17 cell differentiation pathways in the immune response, as well as on iron acquisition mechanisms by the fungus (with the fungal marker *SIDC*). In addition, we compared the infectivity of Tm-ΔSUB6 to that of Tm-ΔKU80 and Tm-SUB6c. The *SUB5* gene was significantly more highly expressed in Tm-ΔSUB6, suggesting a compensatory relationship.

4.1 | *Trichophyton mentagrophytes* TIMM 2789 Strain Is Genotype IV, Like Other Strains Isolated From Rodents, and Exhibits Unique Polymorphisms for the Most Important Typus

Isolates from small rodent infected by or carrying *T. mentagrophytes* are classified here as typus IV, including the TIMM 2789 strain, although strains isolated from guinea pigs have also been associated with typus II* [22, 23]. Rodents are known to be the main reservoir of *T. mentagrophytes*, which causes classical ringworm and/or other skin symptoms, such as alopecia, scaling, erythema and kerions in these animals [35, 36]. Typus III* (which is close to typus IV and II*) is mainly represented by strains isolated from lagomorphs, particularly rabbits [23, 25]. Several cases of human infection have been reported in rabbit breeders, suggesting a zoonotic transmission route [37–39]. *Trichophyton mentagrophytes* has gradually adapted to its hosts, which include rodents, rabbits and other domestic animals, such as carnivorous pets (e.g., cats and dogs). These animals have become primary reservoirs of the dermatophyte. The emergence of genotypes increasingly adapted to humans suggests that a shift in host from animals to humans could represent an ongoing evolutionary trajectory for this dermatophyte [40].

4.2 | Overexpression of Genes Encoding Murine Cytokines and Fungal Markers During Infection With *T. mentagrophytes* TIMM 2789 in Mice

Histopathological results revealed that *T. mentagrophytes* strain TIMM 2789 invaded air follicles as early as Day 4 PI, corroborating the observations from Cambier et al. [11] using a similar mouse model with the *T. mentagrophytes* IHEM 22740 strain (GenBank ITS accession number GU929694). This latter strain was isolated from a human who had been in contact with mice [11, 23].

The results concerning the murine immune response to infection with *T. mentagrophytes* TIMM 2789 show both similarities and notable differences compared with previous studies on

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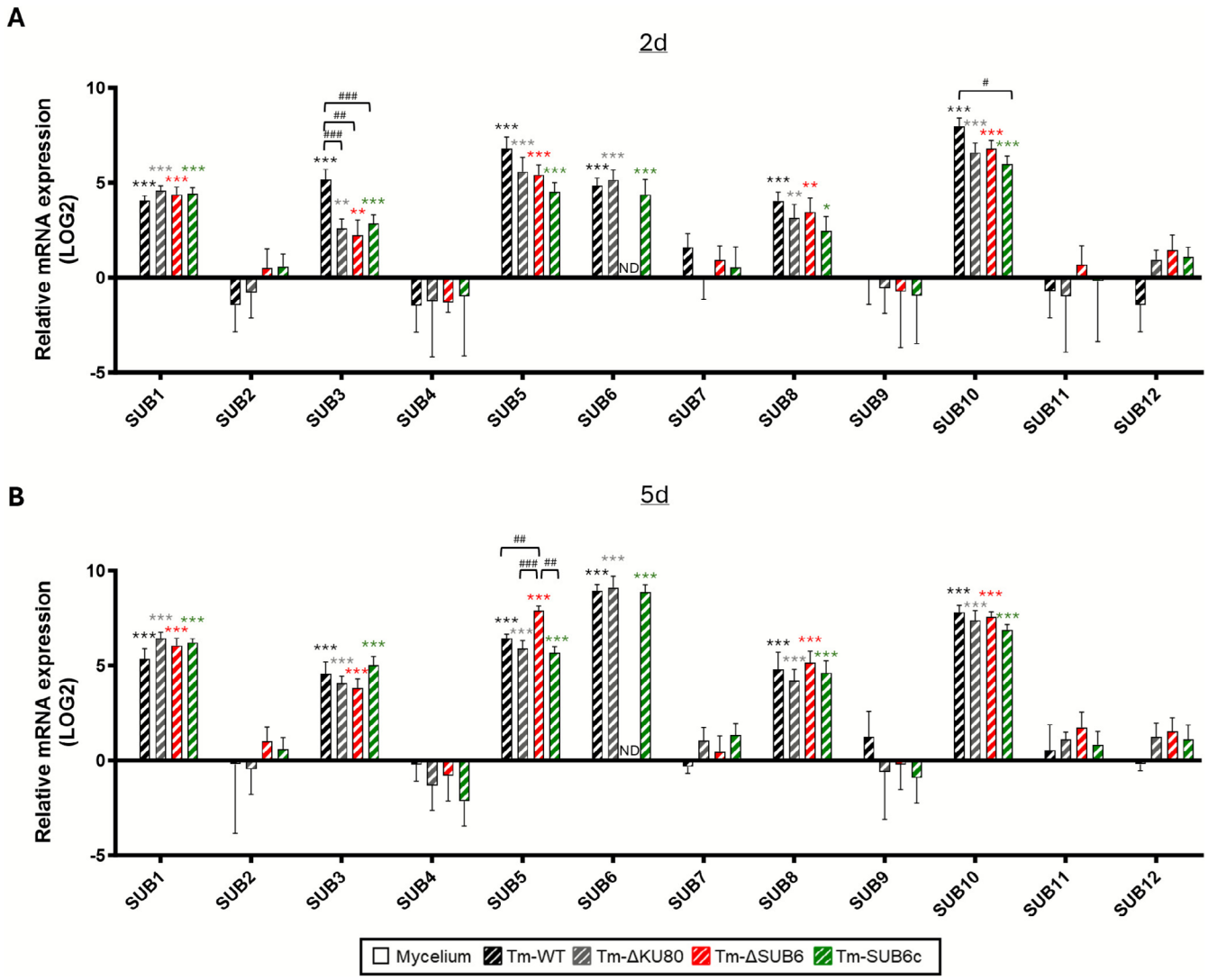

FIGURE 6 | Expression of fungal subtilisins by the *SUB6*-deleted strain (Tm-ΔSUB6) and reference control strains during infection in mice. Mice were infected with *T. mentagrophytes* wild-type (Tm-WT); *KU80*-lacking mutant (Tm-ΔKU80; recipient strain), *SUB6* deleted (Tm-ΔSUB6) or *SUB6* complemented (Tm-SUB6c) strains. The relative messenger RNA (mRNA) expression of fungal subtilisins (SUBs) was assessed by RT-qPCR after total RNA extraction from skin biopsies on (A) Day 2 (2d) and (B) Day 5 (5d) post-infection (PI). The mRNA expression levels were normalised relative to the transcript levels measured in the fungal inoculum (complex [mycelium/hyphae] mixture) used for infection (time point 0), which served as the reference control condition ($n = 6 + \text{SD}$; ANOVA2; */#$p < 0.05$, **/##$p < 0.01$ and ***/###$p < 0.001$; coloured asterisks show significant differences compared with the control condition for each strain; and black hash marks show significant differences between strains; ND, undetected Cq > 45). Complete names and accession numbers of studied genes are listed in Table S4.

dermatophytes. In murine infection models with *T. benhamiae*, *T. mentagrophytes* and *M. canis* [10, 11, 19, 41, 42], the activation of cytokines such as *IFNγ*, *IL-1β*, *IL-6*, *IL-12β* and *TNFα* was also observed. These findings are consistent with our study and support the involvement of the Th1 and Th17 cell differentiation pathways during dermatophyte infection [42]. However, unlike these previous studies, we observed significant overexpression of the *IL-5*, *IL-10* and *IRF5* genes.

IL-10 acts as a key regulator of the immune response during dermatophyte infections. It is initially produced at high levels, suppressing Th1 responses by downregulating *IFNγ* and promoting a Th2 profile, which is less effective in clearing the pathogen [10, 43, 44]. Although limiting pro-inflammatory cytokines such as TNFα to prevent tissue damage, IL-10 levels gradually decline over time, allowing the restoration of a Th1-dominant response essential for fungal clearance [45]. It is worth noting that a previous study examined *IL-10* expression in a murine model infected with *T. mentagrophytes* IHEM 22740, which did not exhibit significant overexpression during infection [11]. However, this strain was initially isolated from a man with dermatophytosis.

Closely associated with IL-10, IL-5 is a signature Th2 cytokine that plays a role in dermatophyte infections. It is involved in the growth, differentiation and activation of eosinophils and contributes to the Th2-mediated immune response [44]. IRF5 plays a central role in immunity against pathogens by mediating the activation of the innate immune responses through the MyD88-dependent Toll-like receptor (TLR) signalling pathway [46–48]. Host TLRs (particularly TLR2 and TLR4) are strongly involved

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in the recognition of dermatophytes during the early stages of infection [4, 49, 50].

In our study of fungal markers, significant overexpression of *ARP2*, *DEUT* and *SIDC* genes was observed. The overexpression of the *ARP2* and *DEUT* genes is comparable to that previously described in other experimental models, both mice and reconstructed human epidermis, using *T. benhamiae* [19]. The *DEUT* gene was also overexpressed in guinea pigs infected with *T. benhamiae* [8]. This suggests that the activation of specific genes by certain dermatophytes is a conserved mechanism, regardless of the hosts. It also highlights the quality of the available experimental models and the potential role of these genes in fungal virulence. Interestingly, the *SIDC* gene, which is involved in the synthesis of intracellular siderophores, showed a significant overexpression in our study. This emphasises the importance of iron acquisition for dermatophytes during infection, which is also a crucial process for other filamentous fungi such as *Aspergillus* or *Scedosporium* species [51–53]. Another interesting observation was the moderate overexpression of the *THAU* gene. Although this gene was found to be highly expressed during *T. benhamiae* infection in guinea pigs [8], our study found it to be moderately overexpressed in mice infected with *T. mentagrophytes* TIMM 2789, suggesting that infection mechanisms may include specific pathways associated with certain dermatophytes and hosts.

4.3 | Overexpression of *SUB5* Could Compensate for the Loss of *SUB6* in Mice Infected With *T. mentagrophytes* TIMM 2789

Deletion of the *SUB6* gene in *T. mentagrophytes* does not significantly affect its virulence in mice, with the Tm-ΔSUB6 strain inducing skin symptoms and microscopic skin lesions similar to those caused by Tm-WT, Tm-ΔKU80 and Tm-SUB6c strains. Analysis of the expression of genes encoding SUBs revealed marked overexpression of the *SUB1*, *SUB3*, *SUB5*, *SUB6*, *SUB8* and *SUB10*, which is consistent with previous observations in experimental models of infection by *T. benhamiae* [19]. Prior to our study, the expression of the *SUB5* gene had never been observed during in vivo infection. The increased expression of *SUB5* in Tm-ΔSUB6 on Day 5 PI suggests that other SUBs may compensate for the loss of the SUB6 protein during infection. As the SUB5 protein does not degrade keratin [54], it may play a later role in the infectious process.

In conclusion, the use of a *T. mentagrophytes* strain isolated from rodents in our mouse model offers a valuable reference framework for the study of dermatophyte infections. Our work provides a better understanding of host–pathogen interactions, as evidenced by the activation of specific Th1, Th2 and Th17 immune responses, as well as the involvement of key fungal markers, notably *SIDC* and *SUB5*, during infection. These observations demonstrate that there is a significant variation in infectious mechanisms depending on the fungal species, the strain and the host used, highlighting the crucial importance of carefully selecting the most appropriate strain for the experimental model used.

Author Contributions

Wilfried Poirier: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, validation, visualization, writing – review and editing, writing – original draft, software, project administration, supervision, resources. **Émilie Faway:** conceptualization, funding acquisition, methodology, supervision, validation, writing – review and editing. **Tsuyoshi Yamada:** conceptualization, data curation, investigation, methodology, validation, writing – review and editing. **Kiyotaka Ozawa:** investigation, methodology. **Françoise Maréchal:** methodology. **Karine Salamin:** methodology. **Romain Vanberg:** methodology, writing – review and editing. **Eléa Denil:** methodology, writing – review and editing. **Michel Monod:** conceptualization, data curation, investigation, methodology, supervision, validation, writing – original draft, writing – review and editing. **Yves Poumay:** conceptualization, funding acquisition, methodology, supervision, validation, writing – review and editing. **Bernard Mignon:** conceptualization, funding acquisition, methodology, project administration, supervision, validation, writing – original draft, writing – review and editing.

Acknowledgements

The authors gratefully acknowledge the histology laboratory of Prof. Nadine Antoine and the technical help provided by Joëlle Piret. This work was supported by the Walloon Region, Walloon Public Service; StratiCELL and Coris BioConcept (MYCEPI, grant number 1910074; TineaDiag, grant number 2410077); and by the Ministry of Education, Culture, Sports, Science and Technology of Japan via a Grant-in-Aid for Scientific Research (20K07054).

Funding

This work was supported by Région Wallonne; Ministry of Education, Culture, Sports, and Technology of Japan.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** myc70141-sup-0001-supinfo.docx.

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