Th1 and Th17 immune responses act complementarily to optimally control superficial dermatophytosis

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TITLE PAGE

Informative title

Th1 and Th17 immune responses act complementarily to optimally control superficial dermatophytosis

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Short title

Th1 and Th17 responses in acute superficial dermatophytosis

Abbreviations

AMP	antimicrobial peptide
CMI	cell-mediated immunity
ELISA	enzyme-linked immunosorbent assay
GATA	GATA binding protein
IFN	interferon
IL	interleukin
LNs	lymph nodes
MPO	myeloperoxidase
Ngp	neutrophilic granule protein
PI	post-inoculation
PMN	polymorphonuclear neutrophil
qRT-PCR	quantitative real-time reverse-transcriptase-PCR
Rag	recombination-activating gene
ROR	retinoic acid receptor-related orphan receptor
T-bet	T-box transcription factor
Th	T helper

UITBC	ultraviolet-irradiated *T. benhamiae* conidia
WT	wild type

ABSTRACT

Dermatophytoses are among the most common fungal infections worldwide but little is known about the immune response in them. By comparing *Trichophyton benhamiae* acute superficial dermatophytosis in WT and Rag2$^{-/-}$ mice, we showed that TCR-mediated immunity is critical for fungal clearance and clinical recovery. In WT mice, CD4+ T-cells isolated from the skin-draining lymph nodes exhibit both Th1 and Th17 differentiation during infection, with regard to produced cytokines or mRNA levels of transcription factors. Using IL-17A- and IFN-γ-deficient mice, we showed that IL-17A and IFN-γ are individually dispensable, but together contribute to the optimal resolution of dermatophytosis. Furthermore, we generated and infected IL-17A and IFN-γ double-deficient mice and showed that both fungal clearance and clinical recovery were much lower in these mice than in single-deficient mice, suggestive of the complementary roles of the two cytokines in dermatophytosis resolution. Thus, our data suggest that TCR-mediated immunity is critical for the optimal control of superficial dermatophytosis and that adaptive immunity is polarized to both Th1 and Th17 responses, with the Th17 antifungal response acting on dermatophyte clearance and the Th1 one being involved in both fungal clearance and Th17-inflammation down-modulation.

INTRODUCTION

Dermatophytosis is a widespread superficial skin mycosis known to affect more than 20–25% of the human population, making it the most common fungal infection (Havlickova et al., 2008; Seebacher et al., 2008; Zhan and Liu, 2016). Among the different fungi causing dermatophytosis in humans, *Trichophyton benhamiae* (formerly *Arthroderma benhamiae*) (de Hoog et al., 2016) is a zoophilic dermatophyte shown to cause acute inflammatory infections (Nenoff et al., 2014b; Zhan and Liu, 2016; Ziegler et al., 2016).

Pathogenic dermatophytes are specialized filamentous fungi that exhibit the capacity to invade keratinized tissues and produce infections that are generally restricted to the stratum corneum, hair, and nails (Weitzman and Summerbell, 1995). Despite their superficial localization, these fungi induce an immune response that remains poorly documented as compared to that observed with other fungal infections. Although the ability of dermatophytes to induce innate response is documented, little is known about the adaptive immune responses against these fungi. Furthermore, the inoculation route in most *in vivo* models used for studying the immune response against dermatophytes fails to mimic the natural epicutaneous infection or even induce observable skin lesions. This drawback limits the relevance of such animal models, leading to conflicting outcomes (Cambier et al., 2017).

The resolution of dermatophytoses is known to be associated with the development of cell-mediated immunity (Calderon and Hay, 1984; Mignon et al., 2008; Schmid-Wendtner and Korting, 2007) with T helper (Th) type 1 polarization (Bressani et al., 2013; Koga et al., 1993a; 1993b; Koga et al., 2001). Despite the crucial role of Th17 response in many other fungal diseases, particularly at mucosal surfaces (Eyerich et al., 2008; Hernandez-Santos and Gaffen, 2012; Hernandez-Santos et al., 2013; Peck and Mellins, 2010; Taylor et al., 2014; Zelante et al., 2009), no study has experimentally documented Th17 response in dermatophytoses.

In this study, using an appropriate mouse model (Cambier et al., 2014), we evidenced that TCR-mediated immunity is critical for the optimal control of acute dermatophytosis and that adaptive immunity is polarized to both Th1 and Th17 responses, wherein these two pathways function in a complementary manner.

RESULTS

TCR-mediated immunity is required for optimal defense against *T. benhamiae* infection

To assess the involvement of TCR-mediated immunity in the fungal clearance and clinical recovery in acute superficial dermatophytosis, we comparatively studied cutaneous *T. benhamiae* infection in recombination-activating gene (rag)2-deficient C57BL/6 ($Rag2^{-/-}$) mice in which lymphocyte development is impaired (Shinkai et al., 1992) and wild-type (WT) littermates. We performed a second infection 63 days after the first one to assess the protective role of a primo-infection in each of these two strains. Mice were sacrificed 3, 7, 14 and 21 days after the first (primary infected mice) or the second (secondary infected mice) infection and their skin were collected for histological analysis and fungal load determination (Figure 1a).

All the infected mice developed clinical signs and histological lesions representative of acute superficial dermatophytosis (Figure 1b and c). The fungal pathogen showed colonization restricted to keratinized skin and follicular structures in both WT and $Rag2^{-/-}$ mice, and was no more invasive in the last strain. In accordance with previous reports (Cambier et al., 2014), the major clinical signs included severe scaling and crusting, which peaked at day 7 post-inoculation (PI). At this time point, the major histological features in both strains were representative of a highly inflammatory state with considerable dermal and perifollicular mononuclear cell infiltration in combination with increased epidermal thickness related to both acanthosis and hyperkeratosis, which were associated with spongiosis and

scattered subcorneal pustules (Figure 1c). In both strains, the cell infiltration was composed of both monomorphonuclear and polymorphonuclear cells. Myeloperoxidase (MPO) activity assays, qRT-PCR evaluation of neutrophilic granule protein (Ngp) levels and direct counting on stained skin sections showed a significant skin infiltration of polymorphonuclear neutrophil (PMN)s at days 3 and 7 PI in both strains of mice, which was significantly higher for the WT mice than for the Rag2$^{-/-}$ ones. At days 14 and 21 PI, WT mice had no more observable infiltrating PMNs, whereas skin of Rag2$^{-/-}$ mice still contained large amount of PMNs (Suppl. Figure S1a, b and c).

In both strains, clinical scores decreased from day 7 to 21 PI (Figure 1d) but were significantly higher for the Rag2$^{-/-}$ group than for the WT controls throughout the experiment. At day 21 PI, WT mice had no more observable lesions, whereas the skin of infected Rag2$^{-/-}$ mice did not heal completely (Figure 1b).

The fungal load progressively decreased from day 3 to 21 PI in the skin of both mouse strains (Figure 1e and f), but was significantly higher in Rag2$^{-/-}$ mice than in WT controls at each time point. Unlike WT mice, Rag2$^{-/-}$ mice showed incomplete elimination of the dermatophyte at day 21 PI.

At the day 0 of secondary infection (day 63 post primary infection), Rag2$^{-/-}$ mice showed no more clinical signs and displayed undetectable fungal loads, as observed from the results of quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). However, fungal cultures from skin samples were deemed positive (4 ± 1.5 CFU/g) (Suppl. Figure S2a, b and c).

The comparison between the primary and secondary infection revealed that the fungal load was significantly lower in WT mice with secondary infection than in those with primary infection at day 3 and day 7 PI. However, in this strain of mice, clinical scores and the time to eliminate the dermatophyte failed to differ between two successive infections. Rag2$^{-/-}$ mice

with secondary infection displayed no difference with respect to both clinical scores (Figure 1d) and fungal loads (Figure 1e and f) when compared with their primary infected counterparts, but showed a slightly lower fungal load at day 3 PI in qRT-PCR analysis (Figure 1f).

Taken together, these data suggest that TCR-mediated immunity significantly contributes to both fungal clearance and clinical recovery in acute superficial dermatophytosis, and exhibits only a slight protective role against re-infection.

The T helper cell subset is polarized towards Th17 and Th1 phenotypes in WT mice infected with *T. benhamiae*

To investigate the adaptive immune response in WT mice infected with *T. benhamiae*, $CD4^+$ T-cells were isolated from the skin-draining lymph node (LN)s at different times PI, and their polarization was determined (Figure 2 and Suppl. Figure S3). In addition, the specificity of the response was evaluated using lymphoproliferative assays on total LNs single-cell suspensions cultured with ultraviolet-irradiated *T. benhamiae* conidia (UITBC) (Figure 3). An increase in the mRNA levels of retinoic acid receptor-related orphan receptor (ROR)γt and T-box transcription factor (T-bet), the major Th17 and Th1 transcription factors, respectively, was observed in $CD4^+$ T-cells isolated from infected mice when compared with those in uninfected controls, whereas the level of GATA-binding protein (GATA)-3, the major Th2 transcription factor, remained unchanged (Figure 2a, b and c). The elevated levels of interleukin (IL)-17A, IL-22, and interferon (IFN)-γ observed in the supernatant of cultured $CD4^+$ T-cells isolated from infected mice (Figure 2d, e and f and Suppl. Figure S3a, b and c), as well as the unchanged IL-4 and IL-10 levels confirmed the Th17 and Th1 polarization of $CD4^+$ T-cells.

Additionally, we demonstrated that this response is specific as ^{3}H-thymidine incorporation and IL-17A and IFN-γ production were significantly higher in UITBC-stimulated cells from skin-draining LNs at day 7 PI than in unstimulated ones. Furthermore, these responses were significantly higher in secondary than in primary infected mice (Figure 3a, b and c).

We investigated whether Th1 and Th17 responses observed in $CD4^{+}$ T-cells from LNs could be evidenced in the infected skin by determining the transcription levels of these cytokines *in situ*. In comparison with the skin samples from control groups, those from the infected groups showed a significant increase in the mRNA levels of IL-17A and IL-22 at day 3 PI, and IL-17A, IL-22, and IFN-γ at day 7 PI (Figure 2g, h and i).

Altogether, these data show that *T. benhamiae* infection induces both specific Th17 and Th1 responses.

While being individually dispensable for fungal elimination, both IL-17A and IFN-γ contribute to optimal control of *T. benhamiae* infection

To evaluate the role of Th17 and Th1 cytokines in fungal clearance and clinical recovery, we compared *T. benhamiae* infection in IL-17A-deficient ($IL\text{-}17A^{-/-}$), IFN-γ-deficient ($IFN\text{-}\gamma^{-/-}$), and WT mice (Figure 4).

As observed in WT controls, infected $IL\text{-}17A^{-/-}$ and $IFN\text{-}\gamma^{-/-}$ mice showed clinical signs and histological lesions representative of acute superficial dermatophytosis. The fungal pathogen colonized and was restricted to keratinized skin and follicular structures (Figure 4a and b).

Unlike $Rag2^{-/-}$ mice, both IL-17A and IFN-γ-deficient mice completely eliminated the dermatophyte and recovered from the infection by day 21 PI (Figure 4c, d and e). While clinical signs in $IL\text{-}17A^{-/-}$ mice were similar to those observed in WT mice, $IFN\text{-}\gamma^{-/-}$ mice

showed exacerbated symptoms at days 3, 7, and 14 PI (Figure 4c). Nevertheless, fungal loads of both deficient strains were significantly higher than those of WT counterparts (at days 3, 7, and 14 PI for IL-17A$^{-/-}$ mice and days 7 and 14 PI for IFN-γ$^{-/-}$ mice; Figure 4d and e).

These data suggest that while IL-17A and IFN-γ are individually dispensable, they both contribute to the optimal resolution of dermatophytosis, with IL-17A acting on dermatophyte clearance and IFN-γ being involved in both fungal clearance and reduction of clinical signs.

Th1 and Th17 responses are upregulated in IL-17A$^{-/-}$ and IFN-γ$^{-/-}$ mice infected with *T. benhamiae*

The clinical recovery of both IL-17A$^{-/-}$ and IFN-γ$^{-/-}$ mice raised the question of the possibility of a compensatory mechanism. Therefore, IL-17A, IL-22, and IFN-γ concentrations were determined in the supernatant of cultured CD4^{+} T-cells isolated from the skin-draining LNs of both the deficient mice (Figure 5 and Suppl. Figure S4).

In uninfected controls, all cytokine levels were below the detection limits of the kit. In infected mice, IL-17A level was significantly higher at days 3 and 7 PI in IFN-γ$^{-/-}$ mice compared to that in WT controls (Figure 5a and Suppl. Figure S4a), and IFN-γ was significantly higher at days 3 and 7 PI in IL-17A$^{-/-}$ mice compared to that in WT controls (Figure 5b and Suppl. Figure S4b).

A significant increase in IL-22 production was observed in both IL-17A$^{-/-}$ and IFN-γ$^{-/-}$ mice, notably at the peak of infection. However, IL-22 upregulation was markedly more significant in IL-17A$^{-/-}$ mice than in IFN-γ$^{-/-}$ mice (Figure 5c and Suppl. Figure S4c).

These data suggest that Th1 and Th17 may compensate for each other during *T. benhamiae* infection at least partially.

IL-17A and IFN-γ have complementary effects during dermatophyte clearance

In order to then investigate whether IL-17A and IFN-γ have complementary effects during disease resolution, mice deficient for both IL-17A and IFN-γ (IL-17A$^{/}$ IFN-γ^{DKO}) were generated and infected with *T. benhamiae* (Figure 6). These animals developed clinical signs and lesions representative of acute superficial dermatophytosis (Figure 6a and b). Their clinical scores showed no significant difference from those observed in IL-17A$^{-/-}$ mice at days 3, 7, and 14 PI, and were significantly lower than those observed in IFN-γ$^{-/-}$ mice at days 3 and 7 PI. Some crusts were still present in IL-17A$^{/}$ IFN-γ^{DKO} mice at day 21 PI, which lead to a significantly higher mean clinical score in these mice when compared with those in both IFN-γ$^{-/-}$ and IL-17A$^{-/-}$ mice (Figure 6c). Furthermore, the fungal load was significantly and markedly higher in IL-17A$^{/}$ IFN-γ^{DKO} mice than in IL-17A$^{-/-}$ and IFN-γ$^{-/-}$ mice at each sampling time point except at day 3 PI, during which it was similar in IL-17A$^{/}$ IFN-γ^{DKO} and IL-17A$^{-/-}$ mice (Figure 6d and e). In addition, IL-22 concentration levels in the supernatant of cultured CD4^{+} T-cells isolated from infected mice were similar in IL-17A$^{/}$ IFN-γ^{DKO} and IL-17A$^{-/-}$ mice. IL-22 levels were significantly higher in IL-17A$^{/}$ IFN-γ^{DKO} than in IFN-γ$^{-/-}$ infected mice at day 7 PI (Figure 6f and Suppl. Figure S5), suggesting that the high level of IL-22 may not compensate for the simultaneous deficiency of both IL-17A and IFN-γ to resolve dermatophytosis.

Altogether, these results suggest that IL-17A and IFN-γ may have complementary effects for optimal fungal clearance during *T. benhamiae* infection.

DISCUSSION

The insufficient knowledge on the global host response against dermatophytosis is notably attributed to the lack of reliable experimental models for dermatophyte infections (Cambier et al., 2017; Shimamura et al., 2012). In our model, the inflammatory signature of clinical signs

and microscopic lesions, fungal colonization of keratinized epidermal and follicular structures and self-healing in WT mouse mimic acute superficial tinea in humans from zoophilic dermatophytes such as *T. benhamiae* (Cambier et al., 2014; Weitzman and Summerbell, 1995). Hence, this model may serve as a powerful tool for the study of the host immune response in acute superficial dermatophytosis.

Using this model, we first showed that TCR-mediated immunity is critical for the optimal control of acute dermatophytosis as $Rag2^{-/-}$ mice presented long lasting infection with higher clinical scores and fungal loads than their WT counterparts. TCR-mediated immunity is achieved by adaptive immune cells but also by innate cells as γδ T-cells. It is well known that CD4+ T-cells are a crucial component of the immune defense against dermatophytes (Heinen et al., 2017) and importance of adaptive immunity is also emphasized by the high incidence of severe or long lasting dermatophyte infections reported in patients with acquired immune deficiency syndrome (Costa et al., 2015; Gupta et al., 2000). However, a recent report showed that adaptive immunity is dispensable for dermatophyte clearance in a mouse model of deep dermatophytosis, a rare condition induced by the anthropophilic pathogen *Trichophyton rubrum* (Dahl and Grando, 1994; Nenoff et al., 2014). Although it is important to consider the differences in experimental design, this previous study and our observation that the fungal load was higher in $Rag2^{-/-}$ mice than in WT controls at day 3 PI but decreased throughout the *T. benhamiae* infection suggest that, even though the adaptive immune response plays a critical role in controlling the disease, the contribution of the innate immune system is also important in host defense against dermatophytes. In mammals, it is generally accepted that the main effector and regulatory mechanisms in the fight against fungal skin infections are (i) increased epidermal turnover, (ii) cytotoxic and phagocytic activity of PMNs and macrophages, as well as (iii) the production of antimicrobial peptides (AMP)s at the site of infection (Blanco and Garcia, 2008; Brasch et al., 2014; Brown, 2011;

Dahl, 1994; Lilic, 2012; Trzeciak-Ryczek et al., 2015; Verma et al., 2014; Wagner and Sohnle, 1995). These three defense mechanisms are induced in dermatophytosis and have been associated with dermatophyte clearance (Heinen et al., 2017). It is also important to mention that during a superficial cutaneous fungal infection, all of these mechanisms can be triggered in a completely innate way. However, if they are insufficient to fight the infection, they will then be amplified by the cell-mediated immunity of the adaptive pathway and especially the Th1 and Th17 pathways. The main effectors cytokines of Th17 cells are IL-17 and IL-22. IL-17 promotes granulopoiesis and the production of PMN-chemoattractant chemokines. IL-17, either alone or in synergy with IL-22, regulates the expression of AMPs and IL-22 regulates the proliferation of keratinocytes (Boniface et al., 2005; Fujita, 2013; Gaffen, 2008; Kolls and Khader, 2010; Liang et al., 2006; Onishi and Gaffen, 2010). Th1 cell-secreted IFN-γ is involved in chemotaxis and is known to be essential for the optimal activation of phagocytic effector cell functions (Schroder et al., 2004). However, IL-17, IL-22 and IFN-γ are produced not only by Th1 and Th17 cells, but also by a number of other cell types including $CD8^+$ T-cells or innate cells such as γδ T-cells, lymphoid tissue inducer cells, innate lymphoid cells and natural killer cells (Annunziato et al., 2015; Ferretti et al., 2003; Gladiator et al., 2013; Isailovic et al., 2015; Montaldo et al., 2015). In addition, IL-17A was reported to be produced by PMNs (Ferretti et al., 2003; Taylor and Pearlman, 2016). Such innate cells can be skin resident or mobilized rapidly in response to infection and do not require prior antigen exposure to be activated. The lack of innate γδ T-cells in $Rag2^{-/-}$ mice could explain the increased fungal burden at day 3 PI compared to WT controls. Furthermore, synthesis of AMPs and attraction of PMNs can even be achieved without the intervention of this innate source of IL-17. For example, keratinocytes in culture are able to sense dermatophytes which leads to the induction of AMPs (Firat et al., 2014) and PMN-chemoattractant chemokines (Achterman et al., 2015; Shiraki et al., 2006; Tani et al., 2007).

Thus this is not surprising to observe a PMN infiltration and a fungal clearance in Rag2$^{-/-}$ mice.

In our model, adaptive immunity played only a minor protective role against re-infection in terms of the time taken for elimination of the dermatophyte and severity of clinical signs. The absence of protection after the primary infection may explain the high recurrence of some dermatophyte infections, at least in part (Gupta et al., 2017; Ilkit and Durdu, 2015). The lower fungal load observed on day 3 PI in secondary infected Rag2$^{-/-}$ mice compared with that in primary infected ones may be related to innate memory, an emerging concept that was recently demonstrated to be mediated by epigenetic reprogramming in innate immune cells (Hamon and Quintin, 2016; Netea et al., 2011; van der Meer et al., 2015). This trained immunity is known to be involved in protection against other fungal infections such as invasive candidiasis (Netea et al., 2015; Quintin et al., 2012).

While the current view is that an efficient host protection against mucosal or cutaneous opportunistic fungal infections requires a coordinated immune response involving Th1 and Th17 cells (Romani, 2011; Wuthrich et al., 2012), the involvement of these two pathways in dermatophytosis had not been demonstrated. Most studies on the adaptive immune response against dermatophytes focused only on the Th1 pathway whose involvement in dermatophytosis is now well documented (Bressani et al., 2013; Koga et al., 1993a; 1993b; Koga et al., 2001; Slunt et al., 1996). The potential involvement of the Th17 pathway in immunity against dermatophytosis is only supported by a extremely limited number of studies (Burstein et al., 2018; Sakuragi et al., 2015). Furthermore, an enhancement in the susceptibility to dermatophyte infections was shown to be related to some immunodeficiencies characterized by the loss of Th17 cell function (Abdel-Rahman, 2016; Engelhardt and Grimbacher, 2012).

In our study, after verifying the specificity of the adaptive responses by UITBC-stimulation in lymphoproliferative assays, we analyzed $CD4^+$ T-cells isolated from the skin-draining LNs, and showed for the first time that the adaptive response in acute superficial dermatophytosis is polarized not only towards Th1 but also towards Th17 response. PMNs infiltration that was more pronounced in infected WT mice than in $Rag2^{-/-}$ is consistent with such cytokine responses. The analysis of the cytokine response in the infected skin of WT mice revealed a significant increase in the mRNA levels of IL-17A, IL-22, and IFN-γ with kinetics similar to that observed in the draining LNs, which could reflect the presence of polarized $CD4^+$ T-cells *in situ*. Indeed, although polarized CD4+ T-cells are not constitutively present in the epidermis, these may migrate into the dermatophyte-infected skin (Brasch and Sterry, 1992; Szepes et al., 1993).

To investigate the individual role of Th1 and Th17 pathways in the control of dermatophytosis, we infected single-deficient IL-17A and IFN-γ mice and found that neither of these cytokines was individually required for the complete elimination of the fungus. However, the impact on the resolution of infection was different in a way that the fungal clearance was less effective and delayed in the presence of IL-17A deficiency, while clinical signs were exacerbated with IFN-γ deficiency.

In our model, we observed an upregulation in the expression of Th17 cytokines (IL-17A and IL-22) and IFN-γ, in IFN-γ- and IL-17A-deficient mice, respectively. This observation may explain why IL-17A and IFN-γ are dispensable for controlling dermatophytosis in single gene-invalidated mice. Reciprocal regulation of Th1 and Th17 developmental pathways is well documented (Lazarevic et al., 2011; Luckheeram et al., 2012) and their mutual compensations have already been described in *Candida* spp. infections under cytokine-deficient conditions (De Luca et al., 2010). We observed an upregulation in IL-22 expression in infected $IL\text{-}17A^{-/-}$ mice, which may reflect a concomitant Th17 amplification. Unlike the

Th1 differentiation mechanism, where the major cytokine IFN-γ acts as the amplifying cytokine, IL-17, the key cytokine of Th17 cells, does not amplify Th17 differentiation (Luckheeram et al., 2012), which may explain that Th17 amplification may also occur during IL-17 deficiency. We have to note that the potential role of IL-17F and other cytokines of the IL-17 family was not explored in this study. Although totally unknown in dermatophyte infection, the role of IL-17F seems to be redundant with the one of IL-17A in defense against *S. aureus* and *Citrobacter rodentium* (Ishigame et al., 2009), but not in cutaneous *Candida albicans* infection where IL-17A$^{-/-}$ mice were shown to be susceptible to the infection despite having normal levels of IL-17F (Kagami et al., 2010).

We observed that the fungal load was higher in IL-17A$^{/}$ IFN-γ^{DKO} than in both IL-17$^{-/-}$ and IFN-γ$^{-/-}$ mice. In addition, the double-deficient mice neither eliminated the dermatophyte nor completely recovered from the infection, which strongly suggests the complementary role of IL-17A and IFN-γ in the optimal control of dermatophytosis.

The exacerbation of clinical signs in IFN-γ$^{-/-}$ mice, but not in IL-17A$^{/}$ IFN-γ^{DKO} mice, may be related to the associated overexpression of IL-17A in case of Th1 deficiency. The role of IL-17A in tissue damage is well documented. High IL-17A concentration may exacerbate inflammatory state, notably by increasing the recruitment of PMNs (Takagi et al., 2017; Zelante et al., 2007). Furthermore, Th1 cells-produced IFN-γ may also act indirectly by inhibiting Th17 expansion, thereby limiting the inflammatory exacerbation and tissue damage.

Although IL-22 has been shown to contribute to antifungal resistance at epithelial surfaces (Sabat et al., 2014), its role, *per se*, in dermatophyte clearance seems to be minor in our model. Although the level of IL-22 in the supernatant of isolated CD4+ T-cells from IL-17A$^{/}$ IFN-γ^{DKO} mice was similar to that from IL-17A$^{-/-}$ mice, IL-17A$^{/}$ IFN-γ^{DKO} mice were unable to clear the infection.

Using a robust mouse model of acute superficial dermatophytosis, we have demonstrated the complementary effect of Th1 and Th17 responses for the optimal control of dermatophytosis, for the first time. Th17 antifungal response acted more efficiently on dermatophyte clearance, whereas Th1 response participated in both fungal clearance and Th17-inflammation down-modulation.

MATERIALS AND METHODS

All housing, breeding and experimental procedures involving mice were approved by the Institutional Animal Ethics Committee (University of Liege, ethics protocol no. 943).

Fungal strain

Trichophyton benhamiae strain (formerly *Arthroderma benhamiae* (de Hoog et al., 2016)), IHEM 20163 (Institute of Hygiene and Epidemiology-Mycology, Brussels, Belgium) was routinely maintained at 27°C on Sabouraud dextrose agar medium (SDA; 2% dextrose, 1% peptone, and 2% agar [all from VWR Scientific Products, San Dimas, CA, USA]). Surface mycelium and conidia were harvested by scraping under sterile conditions, transferring to phosphate-buffered saline (PBS), and filtering through four Miracloth layers (22-25 µm; Calbiochem, La Jolla, CA, USA). Microconidia concentration was determined by serial dilutions on SDA plates.

Animals and experimental infection

C57BL/6 wild-type (WT), C57BL/6 Rag2$^{-/-}$ mice, homozygous IL-17A$^{-/-}$ and IFN-γ$^{-/-}$ mice on C57BL/6 background, and mice that were homozygous for the disruption of both IL-17A and IFN-γ loci (IL-17A$^{/}$ IFN-γ^{DKO}) were epicutaneously inoculated on a shaved 6-cm^2 surface

of dorsal neck and back with 300 µL inoculum comprising 1×10^7 colony forming units (CFUs) of microconidia suspended in 5% (w/w) poloxamer 407 (Sigma, St Louis, USA).

All experimental mice were females between 8 and 16 weeks of age with appropriate littermate controls. Three to six mice were used in each experimental group, and each experiment was repeated at least twice.

See Supplementary Materials and Methods for information on the origin of animals and for additional details on the experimental infection procedure.

Clinical follow-up and scoring of the lesions

Infected mice were blindly monitored at days 3, 7, 14 and 21 PI by two independent examiners using three clinical criteria (erythema, scaling and crusting) and scored using a previously described procedure, with slight modifications (Cambier et al., 2014). See Supplementary Materials and Methods for details on the clinical scoring procedure.

Skin and lymph nodes sampling

Mice were sacrificed and the whole area of infected skin was aseptically collected, weighed, cut into small pieces, and randomly and equally distributed for the following tests: (i) histological procedure, (ii) fungal load determination by CFU counting and qRT-PCR evaluation of chitin synthase (ChiS) levels, (iii) qRT-PCR evaluation of IL-17A, IFN-γ, IL-22 and neutrophilic granule protein (Ngp) levels in the skin, and (iv) myeloperoxidase (MPO) activity measurement.

Lymph node (LN)s draining the inoculated skin surface were resected immediately after euthanasia and pooled for each experimental group. Single-cell suspensions of these LNs were prepared under aseptic conditions. $CD4^+$ T-cells were isolated and cultured. Enzyme-linked immunosorbent assay (ELISA) was performed using the supernatant to determine the

concentrations of IL-17A, IFN-γ, IL-2, IL-4, IL-10, and IL-22. Levels of RORγt, T-bet, GATA-3, IL-17A, IL-22, and IFN-γ were determined by qRT-PCR with the total RNA isolated from $CD4^+$ T-cells.

See Supplementary Materials and Methods for the histological procedure, evaluation of skin PMNs infiltration, evaluation of fungal load by CFU counting, single-cell preparation from LNs, *ex vivo* lymphoproliferative assay and cytokine production determination of cultured total skin-draining LNs cells, $CD4^+$ T-cell isolation and culture, ELISA, and qRT-PCR.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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FIGURE LEGENDS

Figure 1. Adaptive immunity is critical for the optimal control of an experimental superficial acute *T. benhamiae* infection.

Rag2$^{-/-}$ mice and WT littermates were subjected to primary and secondary infections with *T. benhamiae*, with the secondary inoculation being performed 63 days after the first one. (**a**) Schematic timeline of infection, euthanasia, and skin sampling. (**b**) Representative phenotypical appearance during the study period (**c**) representative skin sections at day 7 PI of the infected area. These sections, stained with periodic acid-Schiff and counterstained with hematoxylin, show the fungal colonization of infected mice (yellow arrows), the dermal and perifollicular mononuclear (composed of both monomorphonuclear and polymorphonuclear cells) cell infiltration (black arrows), and the severe increase in the epidermis thickness. Scale bar = 100 µm. (pw: primary infected WT mice; sw: secondary infected WT mice; pr: primary infected Rag2$^{-/-}$ mice). (**d**) Evolution of clinical scores. (**e**, **f**) Fungal loads in the skin were determined by (**e**) CFU counting, and (**f**) qRT-PCR analysis. Data in d, e, and f are means ± standard deviations (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

Figure 2. T helper cell subset is polarized towards Th17 and Th1 phenotypes in WT mice infected with *T. benhamiae*.

Inoculated skin and its draining LNs were sampled at days 0, 3, 7, 14, and 21 PI from primary infected WT mice and their uninfected controls. CD4^{+} T-cells isolated from skin-draining LNs were cultured. (**a, b**, **c**) RORγt, T-bet, and GATA-3 gene expression (relative to GAPDH) was evaluated by qRT-PCR. (**d**, **e**, **f**) Concentrations of IL-17A, IFN-γ, IL-2, IL-4, IL-10, and IL-22 in the culture supernatant were determined by ELISA. The levels of IL-17A, IFN-γ, and IL-22 were below the detection limits of the kits in uninfected mice and those of IL-2, IL-4, and IL-10 were below the detection limits of the kits for both infected and uninfected mice.

(**g**, **h**, **i**) IL-17A, IFN-γ, and IL-22 gene expression (relative to GAPDH) in the skin was evaluated by qRT-PCR analysis. Data are means ± standard deviations (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

Figure 3. The *ex vivo* lymphoproliferative response and cytokine production of total skin-draining lymph nodes cells are specific of *T. benhamiae* in infected WT mice.

Skin-draining LNs were sampled at day 7 PI from primary and secondary infected WT mice and their uninfected controls. Single-cell suspensions from these LNs were cultured for 72 h with PBS, concanavalin-A (concA) or ultraviolet-irradiated *T. benhamiae* conidia (UITBC) and (**a**) ^{3}H-thymidine incorporation was measured (CPM: count per minute) and (**b**, **c**) concentrations of IL-17A and IFN-γ in the culture supernatant were determined by ELISA. Data are means ± standard deviations. (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

Figure 4. IL-17A and IFN-γ contribute to *T. benhamiae* clearance but are dispensable for the resolution of the infection in single-deficient mouse strains.

IL-17A$^{-/-}$ and IFN-γ$^{-/-}$ mice and their WT counterparts were inoculated with *T. benhamiae*. (**a**) Representative phenotypical appearance during the study period and (**b**) representative skin sections at day 7 PI of the infected area. These sections, stained with periodic acid-Schiff and counterstained with hematoxylin, show the fungal colonization (yellow arrows) of epidermal and follicular keratinized structures, the main skin lesions comprising mononuclear cell infiltration (black arrows), and the severe increase in the thickness of epidermis. Scale bar = 100 µm. (**c**) Clinical scores were evaluated. (**d, e**) Fungal loads in the skin were determined by (**d**) CFU counting and (**e**) qRT-PCR analysis. Data in c, d, and e are means ± standard deviations (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

Figure 5. ***T. benhamiae*** **infection induces the overproduction of IL-17A and IFN-γ by CD4$^+$ T-cells in IFN-γ$^{-/-}$ and IL-17A$^{-/-}$ mice, respectively, whereas IL-22 is upregulated in both deficient strains.**

IL-17A$^{-/-}$ and IFN-γ$^{-/-}$ mice and their WT controls were inoculated with *T. benhamiae*. Skin-draining LNs were collected at days 0, 3, 7, 14, and 21 PI and CD4$^+$ T-cells were isolated and cultured. Concentrations in the culture supernatant were determined by ELISA for (**a**) IL-17A (**b**) IFN-γ and (**c**) IL-22. Data are means ± standard deviations (*$P < 0.05$ and ***$P < 0.001$).

Figure 6. IL-17A and IFN-γ have complementary effects during dermatophyte clearance.

Double-deficient IL-17A and IFN-γ (IL-17A$^{/}$ IFN-γ^{DKO}) mice and IL-17A$^{-/-}$, IFN-γ$^{-/-}$ and WT mice were inoculated with *T. benhamiae*. Skin and draining LNs were sampled at days 3, 7, 14, and 21 PI. (**a**) Representative phenotypical appearance of infected and uninfected IL-17A$^{/}$ IFN-γ^{DKO} mice during the study period and (**b**) representative skin sections at day 7 PI of the infected area. These sections, stained with periodic acid-Schiff and counterstained with hematoxylin, show the fungal colonization (yellow arrows) of infected mice, the main skin lesion comprising mononuclear cell infiltration (black arrows), and the severe increase in the epidermis thickness. Scale bar = 100 µm. (**c**) Clinical scores were evaluated. (**d, e**) Fungal loads in the skin were determined by (**d**) CFU counting and (**e**) qRT-PCR analysis. (**f**) IL-22 concentrations were determined by ELISA in the supernatant of cultured CD4$^+$ T-cells. Data in c, d, e, and f are expressed as means ± standard deviations and only the statistical analysis compared with IL-17A$^{/}$ IFN-γ^{DKO} is represented (**$P < 0.01$, and ***$P < 0.001$).

Figure 1

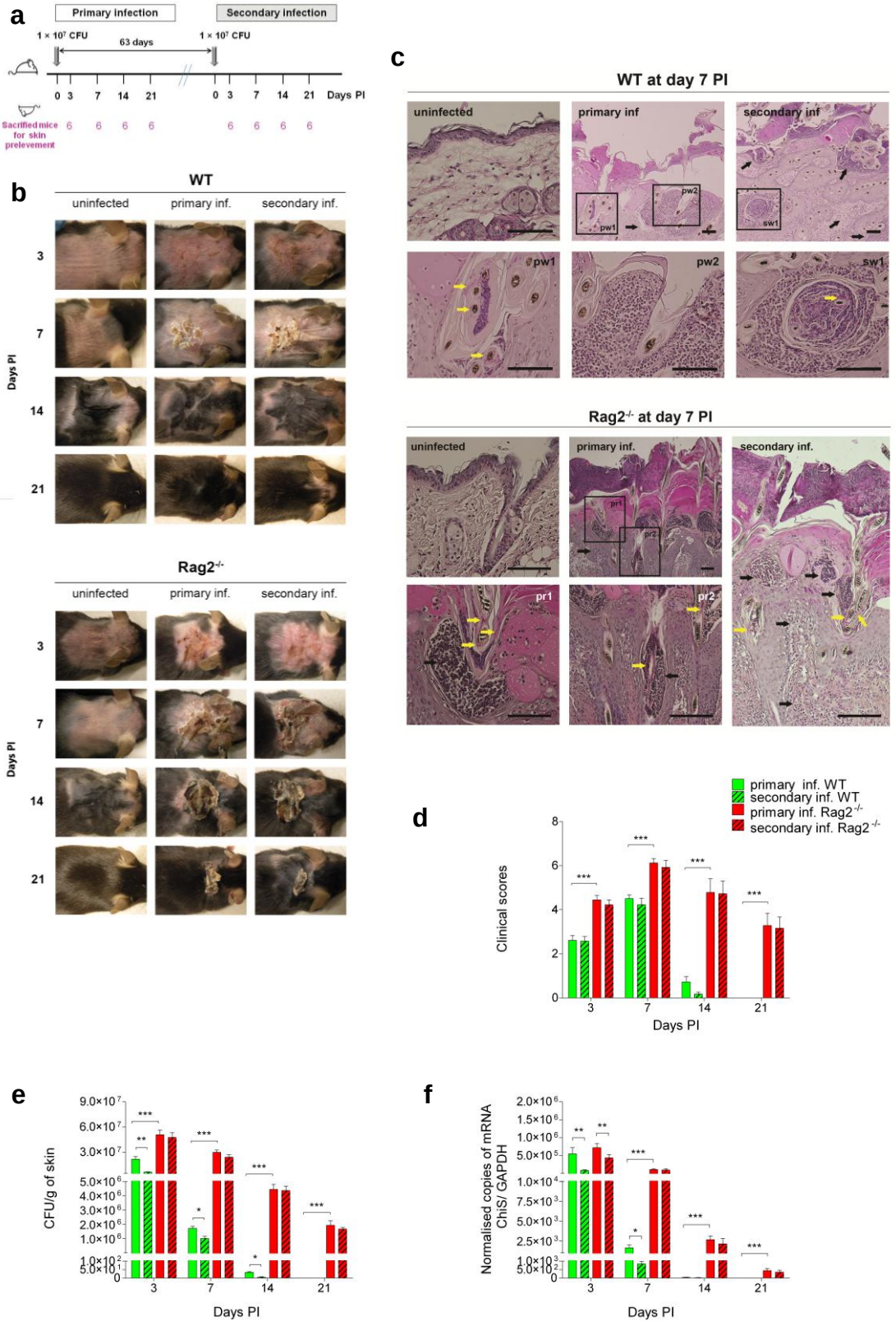

a
Primary infection
Secondary infection
1 × 10⁷ CFU
63 days
1 × 10⁷ CFU
0 3 7 14 21 0 3 7 14 21 Days PI
Sacrified mice for skin prelevement 6 6 6 6 6 6 6 6
b
WT
uninfected
primary inf.
secondary inf.
Days PI
3
7
14
21
Rag2-/-
uninfected
primary inf.
secondary inf.
Days PI
3
7
14
21
c
WT at day 7 PI
uninfected
primary inf
secondary inf
pw1
pw2
sw1
Rag2-/- at day 7 PI
uninfected
primary inf.
secondary inf.
pr1
pr2
d
primary inf. WT
secondary inf. WT
primary inf. Rag2-/-
secondary inf. Rag2-/-
Clinical scores
Days PI
e
CFU/g of skin
Days PI
f
Normalised copies of mRNA ChiS/ GAPDH
Days PI

Figure 2

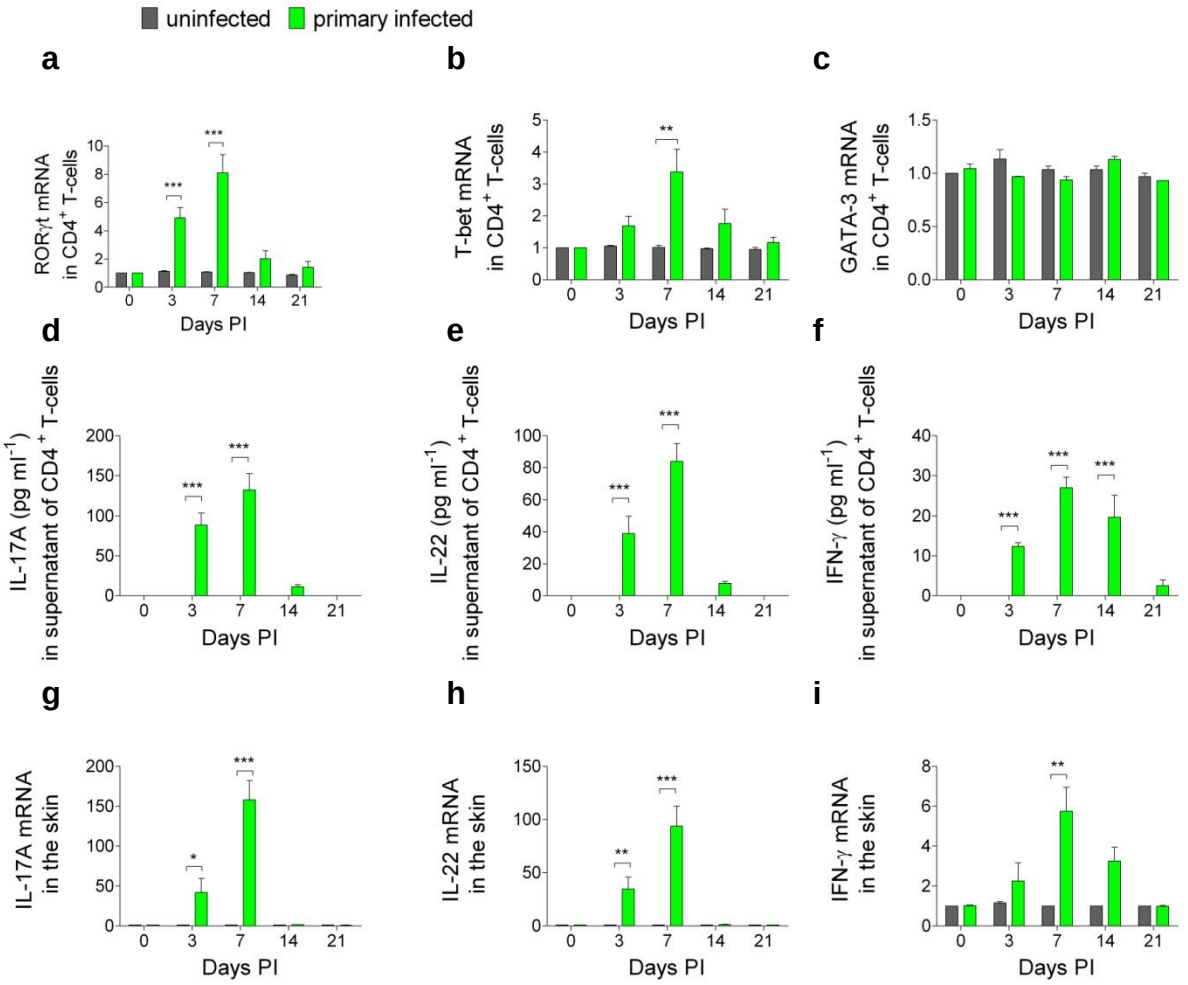

uninfected
primary infected
a
RORγt mRNA in CD4+ T-cells
Days PI
b
T-bet mRNA in CD4+ T-cells
Days PI
c
GATA-3 mRNA in CD4+ T-cells
Days PI
d
IL-17A (pg ml-1) in supernatant of CD4+ T-cells
Days PI
e
IL-22 (pg ml-1) in supernatant of CD4+ T-cells
Days PI
f
IFN-γ (pg ml-1) in supernatant of CD4+ T-cells
Days PI
g
IL-17A mRNA in the skin
Days PI
h
IL-22 mRNA in the skin
Days PI
i
IFN-γ mRNA in the skin
Days PI

Figure 3

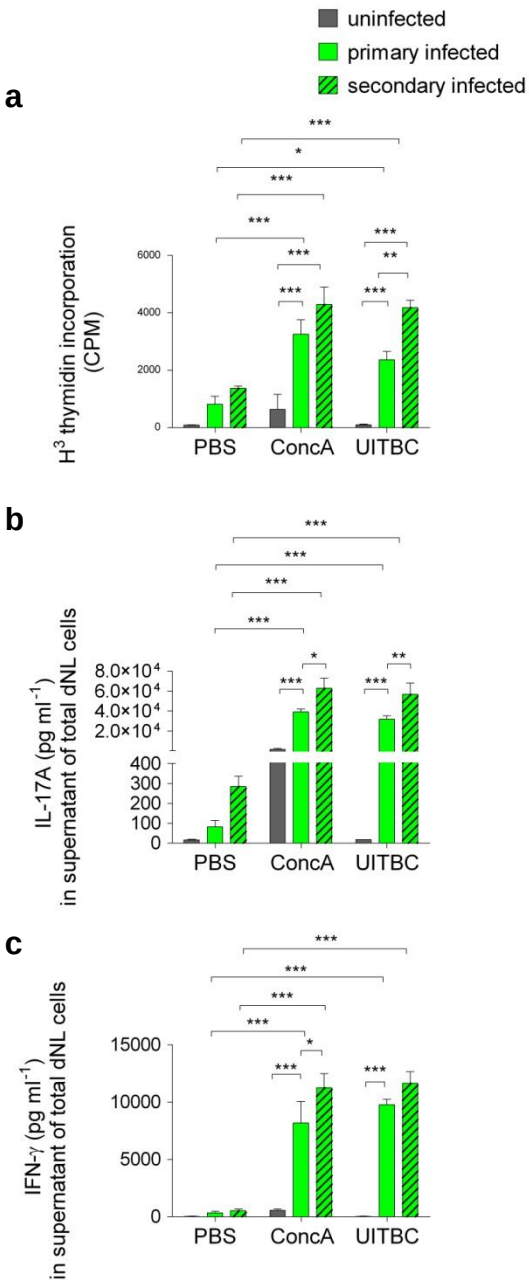

uninfected
primary infected
secondary infected
a
H3 thymidin incorporation (CPM)
6000
4000
2000
0
PBS
ConcA
UITBC
b
IL-17A (pg ml-1) in supernatant of total dNL cells
8.0×10⁴
6.0×10⁴
4.0×10⁴
2.0×10⁴
400
300
200
100
0
c
IFN-γ (pg ml-1) in supernatant of total dNL cells
15000
10000
5000
0

Figure 4

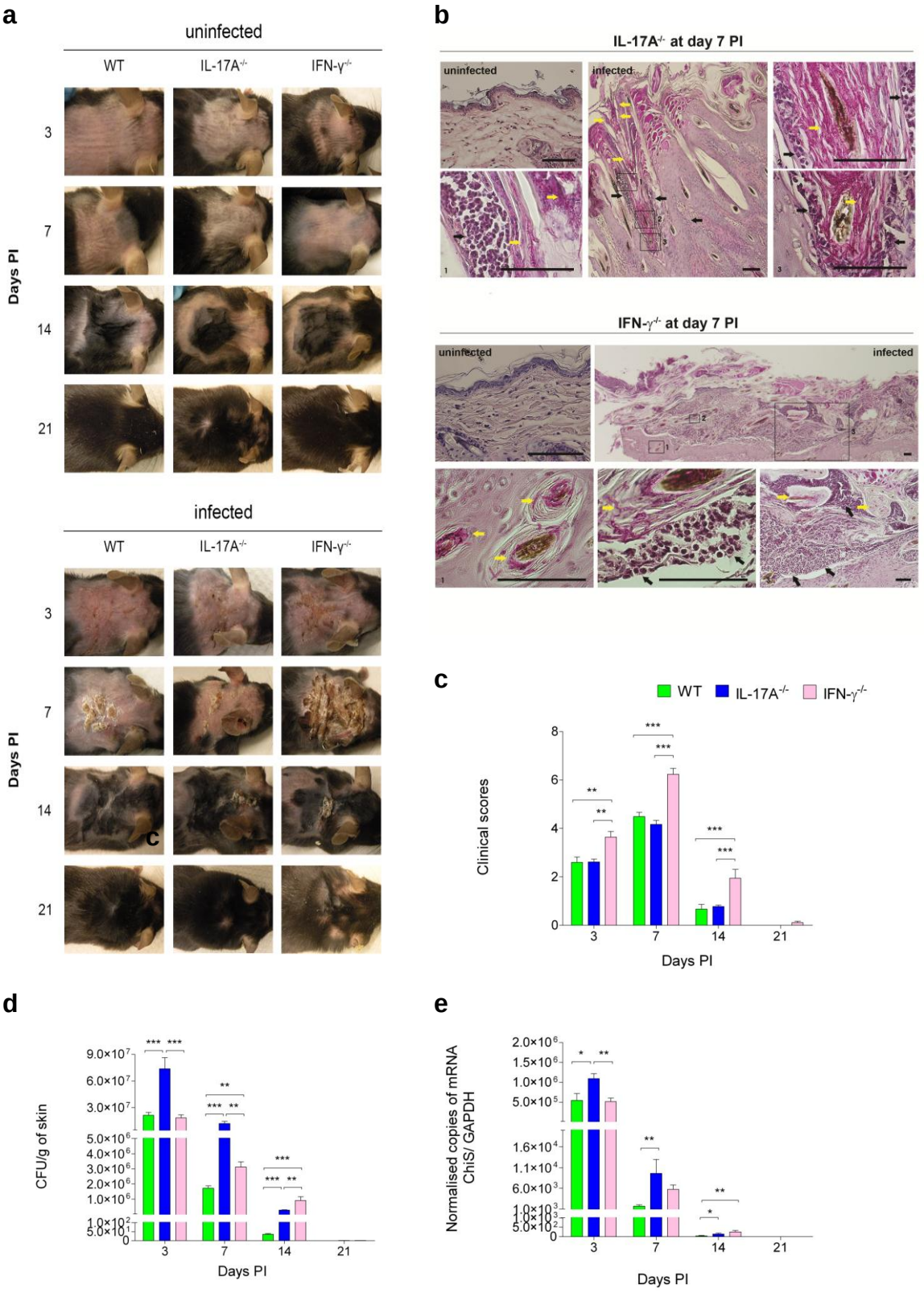

a
uninfected
WT
IL-17A-/-
IFN-γ-/-
Days PI
3
7
14
21
infected
b
IL-17A-/- at day 7 PI
uninfected
infected
IFN-γ-/- at day 7 PI
c
WT
IL-17A-/-
IFN-γ-/-
Clinical scores
Days PI
d
CFU/g of skin
Days PI
e
Normalised copies of mRNA ChiS/ GAPDH
Days PI

Figure 5

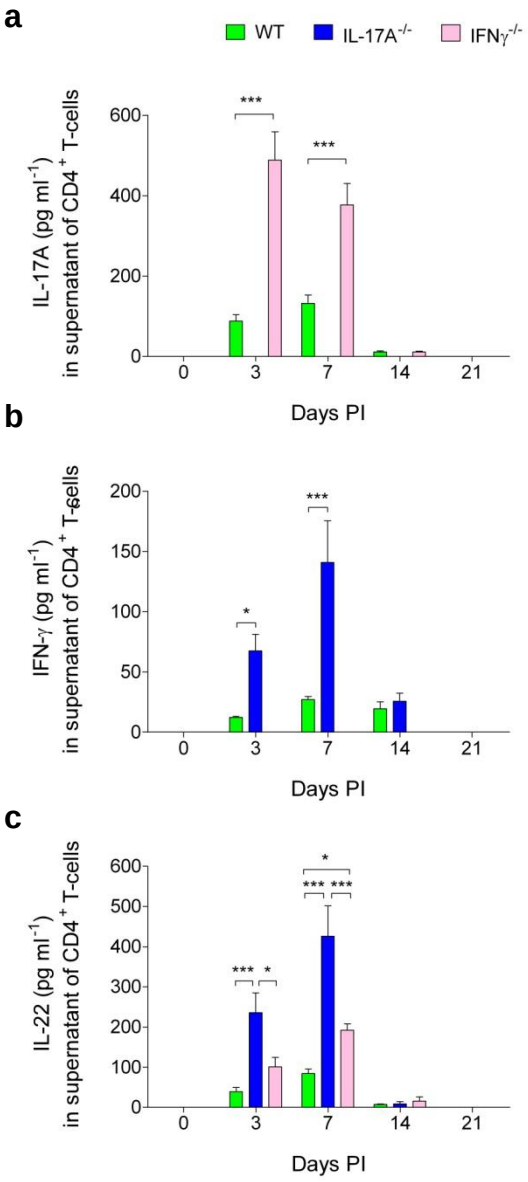
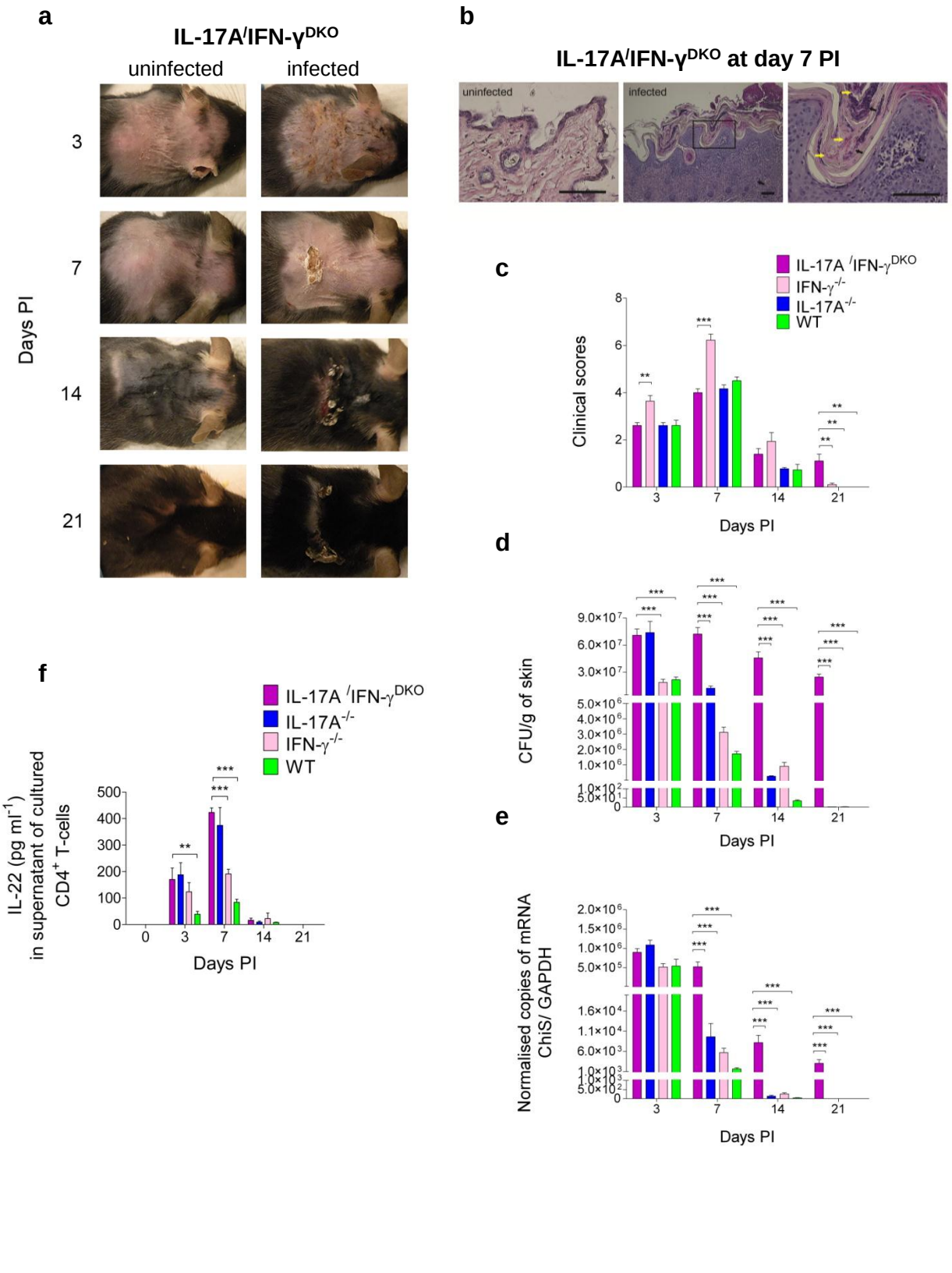

a
WT
IL-17A-/-
IFNγ-/-
IL-17A (pg ml-1) in supernatant of CD4+ T-cells
600
400
200
0
***
***
0
3
7
14
21
Days PI
b
IFN-γ (pg ml-1) in supernatant of CD4+ T-cells
200
150
100
50
0
*
***
0
3
7
14
21
Days PI
c
IL-22 (pg ml-1) in supernatant of CD4+ T-cells
600
500
400
300
200
100
0
*
*** ***
*** *
0
3
7
14
21
Days PI

Figure 6


a
IL-17A/IFN-γDKO
uninfected
infected
Days PI
3
7
14
21
b
IL-17A/IFN-γDKO at day 7 PI
uninfected
infected
c
IL-17A /IFN-γDKO
IFN-γ-/-
IL-17A-/-
WT
Clinical scores
Days PI
d
CFU/g of skin
Days PI
e
Normalised copies of mRNA ChiS/ GAPDH
Days PI
f
IL-17A /IFN-γDKO
IL-17A-/-
IFN-γ-/-
WT
IL-22 (pg ml-1) in supernatant of cultured CD4+ T-cells
Days PI

SUPPLEMENTARY MATERIALS AND METHODS

Origin of animals

C57BL/6 wild-type (WT) mice were purchased from Charles River (Wilmington, MA, USA). C57BL/6 recombination-activating gene *(Rag)2*$^{-/-}$ mice (Shinkai et al., 1992) were generously provided by the *Centre de Développement des Techniques Avancées* (CDTA-CNRS; Orleans, France). Homozygous interleukin (IL)-17A$^{-/-}$ (strain name: STOCK Il17atm1.1 [icre] Stck/J) and interferon (IFN)-γ$^{-/-}$ (strain name: B6.129S7-Ifng/J) mice on a C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME, USA).

To obtain knockout mice that are homozygous for the disruption of both IL-17A and IFN-γ loci (IL-17A$^{/}$ IFN-γ^{DKO}), we mated the aforementioned IL-17A$^{-/-}$ and IFN-γ$^{-/-}$ mice. The resulting double heterozygous IL-17A$^{+/-}$-IFN-γ$^{+/-}$ mice were interbred to attain homozygosity. Experiments were performed with the next generation obtained by breeding these IL-17A$^{-/-}$-IFN-γ$^{-/-}$ (IL-17A$^{/}$ IFN-γ^{DKO}) mice with each other. Mice were genotyped by separated polymerase chain reaction (PCR) analysis of tail DNA using primers and protocols provided by Jackson Laboratory (Master protocol CODE 15019, 002287 and 15003, 016879). The PCR product was separated using the Qiagen QIAxcel Advanced capillary electrophoresis system. Base-pair size of the PCR product was calculated and the genotypes determined using QIAxcel ScreenGel software 1.4.0.

All experimental mice were bred in-house in a specific pathogen-free animal facility. All housing, breeding, and experimental procedures involving mice were approved by the Institutional Animal Ethics Committee (University of Liege, ethics protocol no. 943). Mice were housed separately during the entire infection period.

Experimental infection procedure

Epicutaneous inoculation with *T. benhamiae* microconidia was performed under general anesthesia (medetomidine [1 mg/kg] and ketamine [40 mg/kg]) administered by an intraperitoneal injection. We shaved an area of 6 cm^2 on the skin surface of the neck and back of the animals and abraded it with a 25G needle. A total of 300 µL inoculum comprising 1 × 10^7 colony forming units (CFUs) of microconidia suspended in 5% (w/w) poloxamer 407 (Sigma, St Louis, USA) was gently rubbed onto the skin with a sterile pipette tip. Mice from the control group remained uninfected and received 300 µL of poloxamer only.

Clinical scoring

Both investigators were blinded to the status and strains of mice. For each clinical criterion (erythema, scaling (visible accumulation of loose fragments of the horny layer (stratum corneum) of the skin), and crusting (accumulation of dried exudate, serum, pus, blood, cells, and thick adherent scales on the skin surface)), a score of 0, 1, 2, and 3 (absence, mild, moderate, or severe, respectively) was attributed. A clinical score was then calculated for each mouse by adding the scores for both criteria. Finally, a mean clinical score was calculated for each group of mice.

Histological procedure

Skin samples were routinely processed for mycological and histological examination. These were immediately fixed in 10% neutralized buffered formalin. The tissue was embedded in paraffin and 4-µm-thick sections were stained with hematoxylin and eosin for histopathological evaluation and polymorphonuclear neutrophil (PMN)s direct counting or with periodic acid-Schiff (counterstained with hematoxylin) to assess the fungal invasion in skin structures (all from Sigma, St Louis, USA). Sections were then observed under the

optical microscope Olympus BX51® equipped with an Olympus DP 50® camera (Olympus, Tokyo, Japan).

Evaluation of fungal load by CFU counting

Skin samples were homogenized in 1 mL of sterile phosphate-buffered saline (PBS) using the FastPrep instrument (MP Biomedicals, Santa Ana, CA, USA). Homogenates were diluted to 10-fold in sterile PBS and cultured on Sabouraud dextrose agar (SDA) plates containing 0.05% chloramphenicol and 0.05% cycloheximide (both from Sigma, St Louis, USA). After 3 days of incubation at 27°C, CFUs were counted under the microscope. The results were expressed as CFU per gram of the skin.

Evaluation of skin PMN infiltration

The extent of PMN infiltration in the skin was evaluated by assaying myeloperoxidase (MPO) activity, qRT-PCR evaluation of neutrophilic granule protein (Ngp) levels (see qRT-PCR below) and direct counting on stained (hematoxylin/eosin) skin sections. For this last procedure, PMNs identified in micrographs by their distinctive nuclear morphology in three 40X microscopic fields per skin section were manually counted by two blinded observers. The results were expressed as mean number of PMNs per microscopic field.

Myeloperoxidase (MPO) activity measurement

After thawing, the skin samples (0.1 g of tissue in 1.9 ml of buffer) were homogenized using the TissueLyser system (Qiagen Retsch GmbH, Hannover, Germany) in a pH 4.7 buffer 1 (0.1 M NaCl, 0.02 M Na_2PO_4, 0.015 M Na_2EDTA) (all from Sigma, St Louis, USA), centrifuged at 12000 *g*, at 4°C for 10 minutes. The pellets were resuspended in 100 µl of buffer 1 + 750 µl of a 0.2% NaCl solution + 750 µl of a NaCl 1.6% and glucose 5% solution and thereafter were again homogenized. After further centrifugation (12000 *g* at 4°C for 15 minutes), the pellets were resuspended in 1 ml of a 0.05 M Na_3PO_4 buffer (pH 5.4) containing 0.5%

hexadecyl-trimethylammonium bromide (HTAB; Sigma, St. Louis, USA) and re-homogenized. Samples were then transferred into 1.5-ml microtubes, submitted to three freeze-thaw cycles using liquid nitrogen and then centrifuged at 12000 *g*, at 4°C for 15 minutes to perform the assay. The assay employed 25 µl of 3.4-5.6-tetramethylbenzidine (TMB; Sigma, St. Louis, USA), dissolved in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) at a final concentration of 1.6 mM, 100 µl of H_2O_2, dissolved in phosphate buffer (pH 5.4) containing HTAB at a final concentration of 0.002% vol/vol and 25 µl of sample. The reaction was started at 37°C for 5 minutes in a 96-well microplate by adding the supernatant and the TMB solution. After that, the H_2O_2 solution was added and the microplate was incubated at 37°C for 5 minutes. The reaction was stopped by adding 100 µl of 1 M H_2SO_4 and quantified at 450 nm in a spectrophotometer (Emax; Molecular Devices, Sunnyvale, USA). The results were expressed as relative units to the respective uninfected controls.

Single-cell preparation from lymph nodes

Single-cell suspensions of the lymph nodes were prepared under aseptic conditions. The tissue was mechanically disaggregated through a sterile 70-µm-thick nylon cell strainer (BD Falcon) into sterile Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% (w/w) heat inactivated fetal bovine serum (FBS) (both from Gibco, Renfrewshire, UK). Red blood cells (RBCs) were removed with RBC lysis buffer (Sigma, St Louis, USA) and the cell number of the resulting cells was adjusted with a hemocytometer.

***Ex vivo* lymphoproliferative assay and cytokine production determination of cultured total skin-draining lymph nodes cells**

Lymph node cell density was adjusted to 1×10^6 cells ml^{-1}, and 200 µl of the cell suspensions were then added to a 96-well U-bottom plate (Corning Inc., Corning, USA) and incubated in either PBS (negative control wells), 1 µg concanavalin A (positive control wells) (Sigma, St Louis, USA) or ultraviolet-irradiated *T. benhamiae* conidia (UITBC). Plates were incubated for 3 days at 37°C in an atmosphere containing 5% CO2. For the determination of cytokine production, cell culture supernatants were then harvested and kept frozen at −20°C. For the measure of thymidine incorporation, cells were then incubated with 0.5 µCi/ well of ^{3}H-thymidine (Amersham Biosciences, Piscataway, USA) for 24 h at 37 °C in an atmosphere containing 10% CO2. Cells were harvested onto glass-fiber filters (Skatron, Sterling, VA, USA) using a Skatron cell harvester. The dried filter discs were transferred to vials containing 4 ml of scintillation fluid (Ecoscint A, National Diagnostics, Hessle Hull, UK), and ^{3}H-thymidine incorporation into DNA was quantified for 3 min with a Beckman LS5000 liquid scintillation counter (Beckman Instruments, Fullerton, USA). Data are reported as count per minute (CPM)/animal.

Isolation and culture of CD4$^+$ T-cells

CD4$^+$ T-cells were isolated from the lymph node single-cell suspensions using magnetic-activated cell sorting (MACS) positive selection with CD4 (L3T4) MicroBeads, LS column, and a quadroMACS™ separator according to the manufacturer's protocol (all from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). CD4$^+$ T-cell purity was evaluated by flow cytometry using fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 (IgG2bK) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and was generally over 94%.

CD4$^+$ T-cells were suspended (4×10^6 cells/mL) in RPMI-1640 supplemented with 2 mM l-glutamine, 10% heat-inactivated FBS (all from Gibco, Renfrewshire, UK), 1 mM sodium pyruvate, 100 mM nonessential amino acids, 50 µM 2-mercaptoethanol, 10 mM (4-

(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 50 U/mL penicillin, and 50 µg/mL streptomycin (all from Sigma, St Louis, USA). $CD4^+$ T-cells were stimulated or not with the Mouse T-Activator CD3/CD28 dynabeads (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's protocol. Cells and cell culture supernatants were harvested after 72 h of incubation at 37°C in an atmosphere of 5% CO_2 and kept frozen at −20°C and −80°C until ELISA and qRT-PCR, respectively.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-17A, IFN-γ, IL-2, IL-4, IL-10, and IL-22 in the culture supernatant were determined by ELISA kits (mouse IL-17 A ELISA MAX™ Deluxe Set [BioLegend, San Diego, CA, USA], mouse Th1/Th2 ELISA Ready-SET-Go! [eBioscience, San Diego, CA, USA], and mouse IL-22 ELISA Ready-SET-Go! [eBioscience, San Diego, CA, USA]), as per the manufacturer's instructions. The sensitivity of the assay for IL-17A, IFN-γ, IL-2, IL-4, IL-10, and IL-22 was 8, 15, 2, 4, 30, and 8 pg/mL, respectively.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA from $CD4^+$ T-cells or skin was isolated using RNeasy MiniKit (Qiagen, Valencia, USA) and TRIzol reagent (Invitrogen, Burlington, Canada), respectively, following the manufacturer's protocol. Skin samples were previously homogenized using the TissueLyser system (Qiagen Retsch GmbH, Hannover, Germany). The purified RNA was treated with DNase I (Invitrogen). RNA concentration and purity were assessed with a NanoDrop 2000 (Thermo Scientific). The template cDNA for qRT-PCR was synthesized from RNA by reverse transcription using iScriptTM cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). The mRNA expression of RORγt, T-bet, GATA-3, IL-17A, IL-22, IFN-γ, Ngp and ChiS genes was determined with CFX-96-quantitative RT-PCR system (Bio-Rad Lab) using

iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) and gene-specific primers. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization.

The sequences of oligonucleotide primers were generated using Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and AmplifX program (http://ifrjr.nord.univ-mrs.fr/AmplifX.). The primers were synthesized by Eurogentec (Liege, Belgium) and were RORγt forward 5'-AGTTTGCCAAGCGGCTTTCA-3', reverse 5'-CATTGTAGGCCCTGCACATTCTGA-3'; T-bet forward 5'-TCAACCAGCACCAGACAGAG-3', reverse 5'-AACATCCTGTAATGGCTTGTG-3' ; GATA-3 forward 5'-CTTATCAAGCCCAAGCGAAG-3', reverse 5'-CCCATTAGCGTTCCTCCTC-3'; GAPDH forward 5'-TCAACAGCAACTCCCACTCTTCCA-3', reverse 5'-ACCCTGTTGCTGTAGCCGTATTCA-3'; ChiS forward 5'-ACCTCTGCCAGTGAAATGCACA-3', reverse 5'-TAGACTGGCCACTAGCGAATTCTAGC-3'; IL-17A forward 5'-CTCCAGAATGTGAAGGTCAACCTC-3', reverse 5'-ACTGAGCTTCCCAGATCACAGA-3'; IL-22 forward 5'-TCAGCTCAGCTCCTGTCACATCA-3', reverse 5'-TTCTCGCTCAGACGCAAGCATT-3'; IFN-γ forward 5'-CATCTTGGCTTTGCAGCTCTTCCT-3', reverse 5'-GTCACCATCCTTTTGCCAGTTCCT-3'; Ngp forward 5'-GACTGCGACTTCCTGGAGGATG-3', reverse 5'-GTATCCTCTCGACTGCAATCCCTG-3'

Obtained cycle threshold values were normalized against GAPDH values and the expression difference was calculated using the $2^{(-\Delta\Delta Ct)}$ method, except for ChiS. The level of mRNA at days 3, 7, 14, and 21 was expressed relative to that at day 0. The number of copies of mRNA of ChiS was evaluated using a standard curve generated by serial dilutions of

cDNA standard samples (from 10 to 1 × 10^9) and normalized to that of GAPDH from the corresponding sample.

Statistical analysis

Statistical analysis was performed using the SAS statistical software (version 9.4, SAS Institute Inc., Cary, NC, USA) and GraphPad Prism 5.0 statistical software (GraphPad Software, San Diego, CA, USA). Two-way analysis of variance with Bonferroni's adjustment (Figure 1d, Figure 4c, and Figure 6c) or Kruskal-Wallis and Mann-Whitney tests with Bonferroni's adjustment (Figure 1e and f, Figure 2, Figure 4d and e, Figure 5, Figure 6d and e, Suppl. Figure S1, Suppl. Figure S2, Suppl. Figure S3, Suppl. Figure S4 and Suppl. Figure S5) were used to determine statistical significance (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$). Data in figures represent means ± standard deviations.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1. Comparative infiltration of PMNs in the skin of *T. benhamiae* infected WT and Rag2$^{-/-}$ mice.

Rag2$^{-/-}$ mice and WT littermates were subjected to primary and secondary infections (primary and secondary inf., respectively) with *T. benhamiae*, with the secondary inoculation being performed 63 days after the first one. Inoculated skin samples were collected at days 3, 7, 14, and 21 PI and PMNs infiltration in the skin was evaluated by (**a**) qRT-PCR evaluation of neutrophilic granule protein (Ngp) levels (b) assaying myeloperoxidase (MPO) activity, and (**c**) direct counting on stained (hematoxylin/eosin) skin sections. Data are means ± standard deviations (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

Supplementary Figure S2. Evolution of clinical scores and fungal loads in *T. benhamiae* infected Rag2$^{-/-}$ mice.

Rag2$^{-/-}$ mice were infected with *T. benhamiae* and at days 3, 7, 14, 21, 28, 35, 42, 49, 54 and 63 PI (**a**) clinical scores were evaluated and (**b**, **c**) fungal loads in the skin were determined by (**b**) CFU counting, and (**c**) qRT-PCR evaluation of chitin synthase (ChiS) levels. Data are means ± standard deviations (**$P < 0.01$, and ***$P < 0.001$).

Supplementary Figure S3. IL-17A, IFN-γ and IL-22 concentrations in culture of CD3/CD28-stimulated CD4^{+} T-cells isolated from *T. benhamiae* infected IL-17A$^{-/-}$, IFN-γ$^{-/-}$ and WT mice.

Skin-draining LNs were sampled at days 0, 3, 7, 14, and 21 PI from primary infected WT mice and their uninfected controls. CD4^{+} T-cells isolated from skin-draining lymph nodes were CD3/CD28-stimulated for 72 h. Concentrations of (**a**) IL-17A, (**b**) IFN-γ, and (**c**) IL-22

in the culture supernatants were determined by ELISA. Data are means ± standard deviations (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

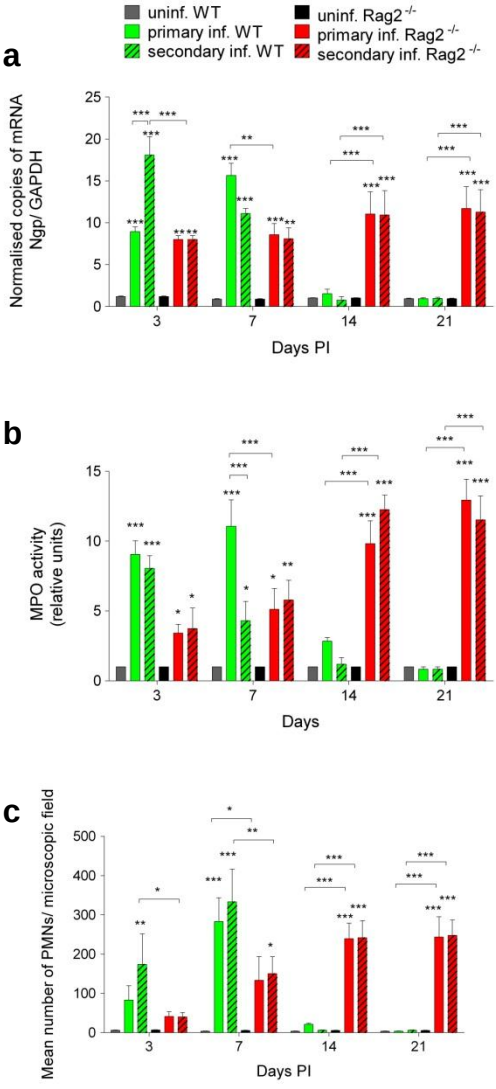
Supplementary Figure S4. IL-17A, IFN-γ and IL-22 concentrations in culture of CD3/CD28-stimulated CD4$^+$ T-cells isolated from *T. benhamiae* infected IL-17A$^{-/-}$, IFN-γ$^{-/-}$ and WT mice.

IL-17A$^{-/-}$ and IFN-γ$^{-/-}$ mice and their WT controls were inoculated with *T. benhamiae*. Skin-draining LNs were collected at days 0, 3, 7, 14, and 21 PI and CD4$^+$ T-cells were isolated and cultured with CD3/CD28-stimulation for 72 h. Concentrations in the culture supernatants were determined by ELISA for (**a**) IL-17A (**b**) IFN-γ and (**c**) IL-22. Data are means ± standard deviations (*$P < 0.05$ and ***$P < 0.001$).

Supplementary Figure S5. IL-22 production by CD3/CD28-stimulated CD4$^+$ T-cells isolated from *T. benhamiae* infected IL-17A$^/$ IFN-γ^{DKO}, IL-17A$^{-/-}$, IFN-γ$^{-/-}$ and WT mice.

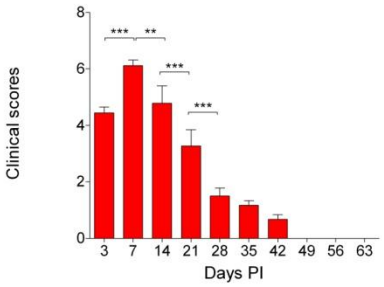
Double-deficient IL-17A and IFN-γ (IL-17A$^/$ IFN-γ^{DKO}) mice and IL-17A$^{-/-}$, IFN-γ$^{-/-}$ and WT mice were inoculated with *T. benhamiae*. Skin-draining LNs were collected at days 0, 3, 7, 14, and 21 PI and CD4$^+$ T-cells were isolated and cultured with CD3/CD28-stimulation for 72 h. IL-22 concentrations in the culture supernatant were determined by ELISA. Data are means ± standard deviations (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

Supplementary Figure S1



Supplementary Figure S2

a



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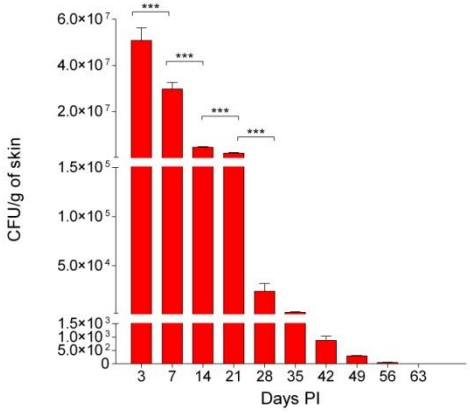
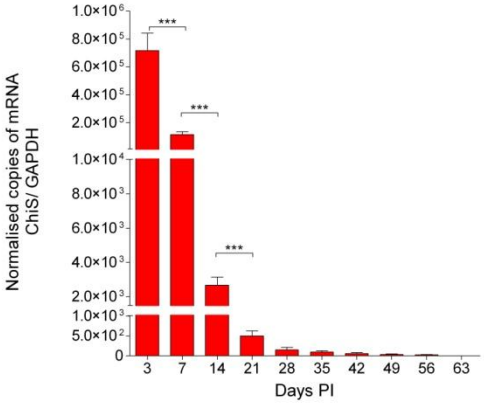
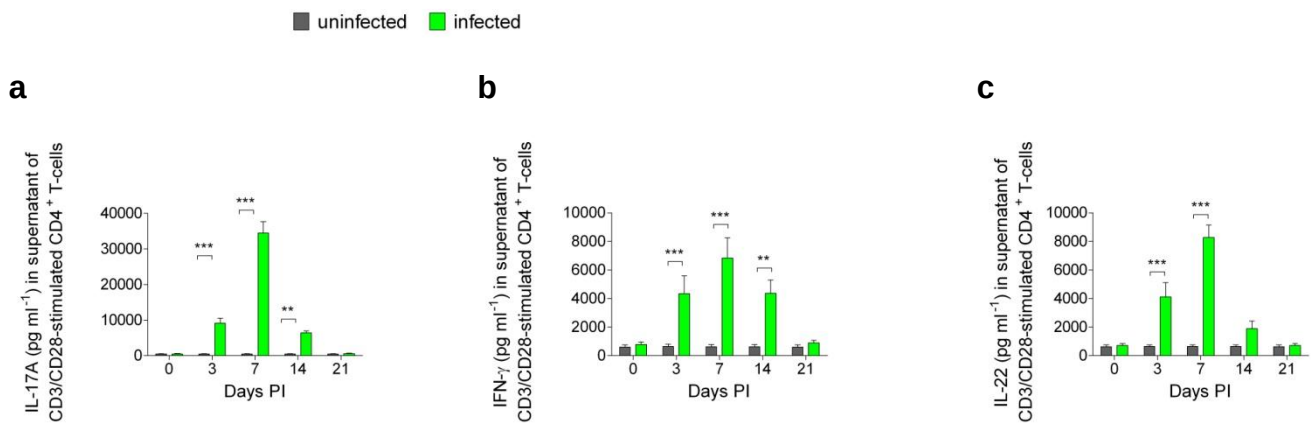


c



Supplementary Figure S3



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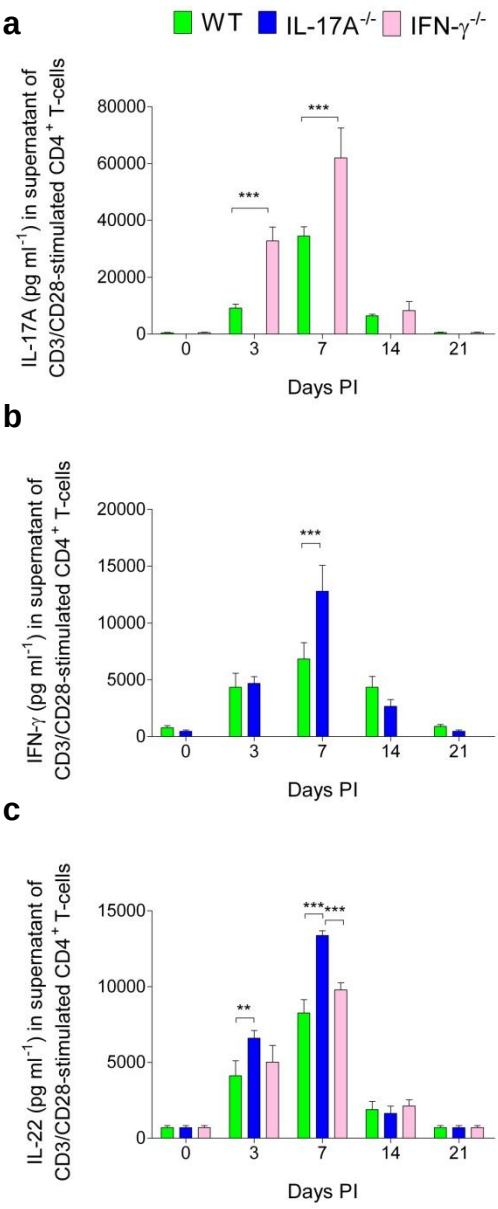
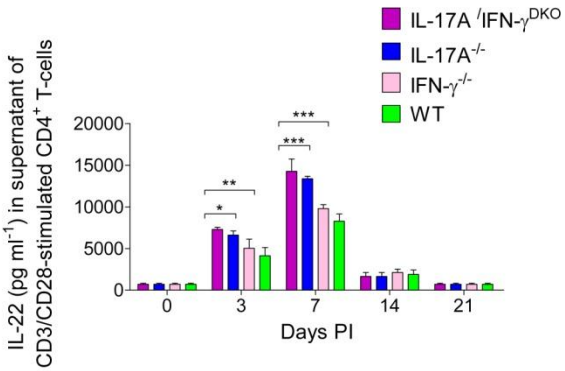


Supplementary Figure S5



Faculty of Veterinary Medicine
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To Mark C. UDEY,
Editor of the *Journal of Investigative Dermatology*

Author's Response to Decision Letter for (MS# JID-2018-0277)

Editor comments: The topic of this paper is of interest, but all of the reviewers and the Section/Deputy Editor had concerns. The authors should respond to each of the points raised by the reviewers and utilize the comments of the Section/Deputy Editor as a guide to performing the necessary experiments in preparing the revision.

On behalf of my co-authors, I would like to thank you for your interest and for giving us the opportunity to revise our manuscript. We appreciate editor and reviewers very much for their positive and constructive comments and suggestions on our manuscript entitled "Th1 and Th17 immune responses act complementarily to control superficial dermatophytosis". We carefully considered your comments, as well as those offered by the three reviewers, and all of them have been addressed point-by-point as follows. Herein, we explain how we revised the paper based on those comments and recommendations. The changes in the manuscript have been highlighted in yellow.
Yours sincerely,

On behalf of all co-authors,

HEINEN Marie-Pierre

Section/Deputy Editor: 1

Comments to the Author:

There is enthusiasm for this manuscript though several technical concerns must be addressed: Antigen-specific responses should be evaluated as per reviewer #1. Ex vivo flow analysis of ag-specific T cells should be informative. The lack of a requirement for Type-17 inflammation is surprising. The relative contribution of TCR ab and TCR gd should be specifically explored. There may also be redundancy between IL-17A and IL-17F leading to the observed phenotype. IFNg-/- mice may also have a compensatory increased Type 17 response. This should be examined on a single cell basis.

We are very pleased by the enthusiasm of the deputy Editor. All of the reviewers' suggestions and propositions have been addressed point-by-point in the following sections.

In summary:

- We added data highlighting the antigen-specificity of the observed response (Figure 3), data on evaluation of polymorphonuclear neutrophil (PMN)s infiltration in the skin of infected WT and Rag2$^{-/-}$ mice (Suppl. Figure S1), data on evolution of clinical scores and fungal loads in *T. benhamiae* infected Rag2$^{-/-}$ mice after day 21 PI (Suppl. Figure S2) and data on cytokine production in culture of CD3/CD28-stimulated CD4+ T-cells (Suppl. Figure S3, Suppl. Figure S4, and Suppl. Figure S5).
- We modified the structure of the discussion and emphasized that the host's response against dermatophytes includes both innate and acquired responses. We clearly stated that the innate response includes not only IL-17 and IFN-γ-dependent mechanisms, but also IL-17- and IFN-γ-independent mechanisms, which could explain our observation that differences in the clinical scores in the various strains of mice, including the double KO ones, are not clear cut.
- Concerning the interpretation of the data from Rag2$^{-/-}$ mice, we substituted "adaptive immunity" by "TCR-mediated immunity" and specified that Rag2$^{-/-}$ mice lack αβ cells and γδ T-cells. We also highlighted the intervention of the innate response in dermatophytosis.
- Additional data on infiltrating PMNs in skin of infected WT and Rag2 $^{-/-}$ mice allow to highlight the PMNs innate cutaneous infiltration (independent of γδ T-cells) (Suppl. Figure S1). We also have data showing both PMNs infiltration and antimicrobial peptides expression in the skin of all the mentioned deficient strains of mice. Nevertheless, we chose not to publish them in this manuscript, in the sole purpose not to complicate the message.
- We also warned the reader on the unexplored possible role of IL-17F and other cytokines of the IL-17 family.

- The compensatory increased type 17 response in IFN-γ deficient mice was already mentioned in our results and in our discussion, but we now added data on cytokine production in culture of CD3/CD28-stimulated CD4+ T-cells to strengthen the value of the data (Suppl. Figure S4).
- Regarding to the suggestion of a better characterization of the immune response at the cellular level through the use of flow cytometry and/or other deficient strains of mice, although we find this very relevant, we could not perform additional dedicated experiments because of a lack of remaining adequate samples and funding. Nevertheless, and accordingly we have interpreted our data in a more nuanced way to avoid any erroneous conclusions.

Reviewer Comments* and *Responses:

Reviewer 1

Comments to the Author

This manuscript by Marie-Pierre et al. establishes a murine Tricophyton benjamia infection model to study the cutaneous immune response to dermatophyte infections. This article contributes significantly to the readers of an investigative dermatology journal with new techniques and insights. The manuscript does have notable areas requiring improvement that should be addressed before eventual publication into the JID.

Strengths

1. While dermatophytosis is relatively common, there is relative deficit of reductionist study models to investigate the immune response. Authors establish a murine dermatophyte infection model using Tricophyton spp. that is not easy and could be of use and interest to the readers of an investigative dermatology journal.

2. Authors perform a relative solid characterization of the infection model via clinical scoring, histology, immune analysis and use of cytokine deficient mice.

3. The dual requirement for IL-17A/IFN-g in controlling dermatophytosis would be novel and significant addition.

Authors would like to thank the Reviewer 1 for considering our manuscript very interesting as well as for her/his positive and very constructive comments, which undoubtedly helped us to improve the quality of the manuscript. Please, see below our answers to your queries and comments.

Areas requiring attention

1.

The methods of investigating the immune response can be significantly improved. Transcript analysis by mRNA and elisa of CD4+ T cell cultures are not appropriate since they do not address the role of antigen specificity. To overcome this, authors should utilize well-established and doable flow cytometry approaches to elucidate what is occurring with the immune response. CD4+ T cells can be isolated and re-stimulated with heat killed conidia.

We totally agree with this comment. We therefore added new data to improve the investigation of the immune response, especially to address the question of antigen specificity. As we think that for an optimal specific re-stimulation of CD4+ T-cells with the antigen, the latter has to be presented by APC, we used total skin-draining lymph node (LN)s cells to evaluate *ex vivo* both lymphoproliferative

response and cytokine production of cultured LNs cells. Tritiated (^{3}H)-thymidine incorporation as well as IL-17A and IFN-γ production were determined after re-stimulation (with PBS, concanavalin-A or ultraviolet-irradiated *T. benhamiae* conidia (UITBC)) of LNs cells isolated from uninfected, primary and secondary infected WT mice *(*Figure 3). Results show the *T. benhamiae* specificity of the studied response. Furthermore, in addition to results on concentrations of IL-17A, IFN-γ, IL-2, IL-4, IL-10, and IL-22 that were determined in the culture supernatant of CD4$^+$ T-cells isolated from skin-draining LNs (Figure 2d, e and f, Figure 5, and Figure 6f), we provide new data on the concentration of these cytokines in the supernatant of CD3/CD28-stimulated CD4+ T-cells (Suppl. Figure S3, Suppl. Figure S4 and Suppl. Figure S5).

These additional data were implemented as follows.

- in Results: p8 in: The T helper cell subset is polarized towards Th17 and Th1 phenotypes in WT mice infected with *T. benhamiae*

  To investigate the adaptive immune response in WT mice infected with *T. benhamiae*, CD4$^+$ T-cells were isolated from the skin-draining lymph node (LN)s at different times PI, and their polarization was determined (Figure 2 and Suppl. Figure S3). In addition, the specificity of the response was evaluated using lymphoproliferative assays on total LNs single-cell suspensions cultured with ultraviolet-irradiated *T. benhamiae* conidia (UITBC) (Figure 3). [...] The elevated levels of interleukin (IL)-17A, IL-22, and interferon (IFN)-γ observed in the supernatant of cultured CD4$^+$ T-cells isolated from infected mice (Figure 2d, e and f and Suppl. Figure S3a, b and c), as well as the unchanged IL-4 and IL-10 levels confirmed the Th17 and Th1 polarization of CD4$^+$ T-cells.

  Additionally, we demonstrated that this response is specific as ^{3}H-thymidine incorporation and IL-17A and IFN-γ production were significantly higher in UITBC-stimulated cells from skin-draining LNs at day 7 PI than in unstimulated ones. Furthermore, these responses were significantly higher in secondary than in primary infected mice (Figure 3a, b and c). […]

  Altogether, these data show that *T. benhamiae* infection induces both specific Th17 and Th1 responses.

- In Discussion: p15: In our study, after verifying the specificity of the adaptive responses by UITBC-stimulation in lymphoproliferative assays, we analyzed CD4$^+$ T-cells isolated from the skin-draining LNs, and showed for the first time that the adaptive response in acute superficial dermatophytosis is polarized not only towards Th1 but also towards Th17 response.

- In Figure Legends:

  - Figure 3. The *ex vivo* lymphoproliferative response and cytokine production of total skin-draining lymph nodes cells are specific of *T. benhamiae* in infected WT mice.

    Skin-draining LNs were sampled at day 7 PI from primary and secondary infected WT mice and their uninfected controls. Single-cell suspensions from these LNs were cultured

for 72 h with PBS, concanavalin-A (concA) or ultraviolet-irradiated *T. benhamiae* conidia (UITBC) and **(a)** ^{3}H-thymidine incorporation was measured and (**b**, **c**) concentrations of IL-17A and IFN-γ in the culture supernatant were determined by ELISA. Data are means ± standard deviations. (**P* < 0.05, ***P* < 0.01, and ****P* < 0.001).

- In Materials and Methods: See Supplementary Materials and Methods for the histological procedure, evaluation of skin PMNs infiltration, evaluation of fungal load by CFU counting, single-cell preparation from LNs, *ex vivo* lymphoproliferative assay and cytokine production determination of cultured total skin-draining LNs cells, $CD4^+$ T-cell isolation and culture, ELISA, and qRT-PCR.
- In Supplementary Figure Legends:
  - Supplementary Figure S3. IL-17A, IFN-γ and IL-22 concentrations in culture of CD3/CD28-stimulated $CD4^+$ T-cells isolated from *T. benhamiae* infected IL-17A$^{-/-}$, IFN-γ$^{-/-}$ and WT mice.
    Skin-draining LNs were sampled at days 0, 3, 7, 14, and 21 PI from primary infected WT mice and their uninfected controls. $CD4^+$ T-cells isolated from skin-draining LNs were cultured with CD3/CD28-stimulation for 72 h. Concentrations of (a) IL-17A, (b) IFN-γ, and (c) IL-22 in the culture supernatants were determined by ELISA. Data are means ± standard deviations (**P* < 0.05, ***P* < 0.01, and ****P* < 0.001).
  - Supplementary Figure S4. IL-17A, IFN-γ and IL-22 concentrations in culture of CD3/CD28-stimulated $CD4^+$ T-cells isolated from *T. benhamiae* infected IL-17A$^{-/-}$, IFN-γ$^{-/-}$ and WT mice.
    IL-17A$^{-/-}$ and IFN-γ$^{-/-}$ mice and their WT controls were inoculated with *T. benhamiae*. Skin-draining LNs were collected at days 0, 3, 7, 14, and 21 PI and $CD4^+$ T-cells were isolated and cultured with CD3/CD28-stimulation for 72 h. Concentrations in the culture supernatants were determined by ELISA for (a) IL-17A (b) IFN-γ and (c) IL-22. Data are means ± standard deviations (**P* < 0.05 and ****P* < 0.001).
  - Supplementary Figure S5. IL-22 production by CD3/CD28-stimulated $CD4^+$ T-cells isolated from *T. benhamiae* infected IL-17A$^{/}$ IFN-γ^{DKO}, IL-17A$^{-/-}$, IFN-γ$^{-/-}$ and WT mice.
    Double-deficient IL-17A and IFN-γ (IL-17A$^{/}$ IFN-γ^{DKO}) mice and IL-17A$^{-/-}$, IFN-γ$^{-/-}$ and WT mice were inoculated with *T. benhamiae*. Skin-draining LNs were collected at days 0, 3, 7, 14, and 21 PI and $CD4^+$ T-cells were isolated and cultured with CD3/CD28-stimulation for 72 h. IL-22 concentrations in the culture supernatant were determined by ELISA. Data are means ± standard deviations (**P* < 0.05, ***P* < 0.01, and ****P* < 0.001).
- In Supplementary Materials and Methods:
  - Isolation and culture of $CD4^+$ T-cells: […] $CD4^+$ T-cells were stimulated or not with the Mouse T-Activator CD3/CD28 dynabeads (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's protocol. […]

- *Ex vivo* lymphoproliferative assay and cytokine production determination of cultured total skin-draining LNs cells

  Lymph node cell density was adjusted to 1 X 10^6 cells ml^{-1}, and 200 µl of the cell suspensions were then added to a 96-well U-bottom plate (Corning Inc., Corning, USA) and incubated in either PBS (negative control wells), 1 µg concanavalin-A (positive control wells) (Sigma, St Louis, USA) or ultraviolet-irradiated *T. benhamiae* conidia (UITBC). Plates were incubated for 3 days at 37°C in an atmosphere containing 5% CO2. For the determination of cytokine production, cell culture supernatants were then harvested and kept frozen at −20°C. For the measure of thymidine incorporation, cells were then incubated with 0.5 µCi/ well of ^{3}H-thymidine (Amersham Biosciences, Piscataway, USA) for 24 h at 37 °C in an atmosphere containing 10% CO2. Cells were harvested onto glass-fiber filters (Skatron, Sterling, VA, USA) using a Skatron cell harvester. The dried filter discs were transferred to vials containing 4 ml of scintillation fluid (Ecoscint A, National Diagnostics, Hessle Hull, UK), and ^{3}H-thymidine incorporation into DNA was quantified for 3 min with a Beckman LS5000 liquid scintillation counter (Beckman Instruments, Fullerton, USA). Data are reported as count per minute (CPM)/animal.

A tetramer for an immunodominant epitope for Tricophyton spp. can also be used but this may be out of the scope of the manuscript (Wutrich et al., Cell Host & Microbe 2015.)

The use of an immunodominant epitope for *Trichophyton* spp. could be of interest but indeed probably out of the scope of this manuscript. We should add that in the aforementioned lymphoproliferative assays we successfully re-stimulated the cells with both concanavalin-A (positive test) and UIBTC (Figure 3). The choice of a crude structural antigen (irradiated conidia) rather than recombinant secreted antigens is based on preliminary experiments showing that tested *Trichophyton* endo- and exoproteases induced weaker responses than UIBTC (data not shown).

At the very least it would be nice to get flow cytometry with PMA/ionomycin from lymph nodes and skin to obtain information as to the cellular source of cytokines (i.e. is the source for IL17/IFNg coming from the same source, are gd T cells involved, etc

As mentioned above, for technical reasons, we unfortunately cannot perform additional experiments to better characterize the cytokine response at the cellular level using flow cytometry and/or other deficient strains of mice. However, we totally agree with the Reviewer that IL-17 and IFN-γ are produced by a number of adaptive and innate immune cells (see below).

As a consequence, we slightly modified the discussion as follows: p14… The analysis of the cytokine response in the infected skin of WT mice revealed a significant increase in the mRNA levels of IL-17A, IL-22, and IFN-γ with kinetics similar to that observed in the draining LNs, which could reflect the presence of polarized $CD4^+$ T-cells *in situ*. Indeed, although polarized CD4+ T-cells are not constitutively present in the epidermis, these may migrate into the dermatophyte-infected skin (Brasch and Sterry, 1992; Szepes et al., 1993).

We should add that at this point of the discussion we have already warned the reader of a possible innate source of these cytokines.

2.

I am concerned about the interpretation of the data. For example, authors note that adaptive immunity is required given the increased fungal burden in Rag deficient mice. However, rag deficient mice also have a defect in IL-17 producing gd T cells of the skin and other organs and rag is important in the function of innate cells. Authors demonstrate that Rag -/- mice have increased fungal burden at day 3 compared to controls, which suggest an innate defect and then also demonstrate that there is no difference in the rag-/- in CFU between primary and secondary infections, which makes it seem like there is no effect of the adaptive immune response (there would be an increased fungal burden in the secondary infection compared to the primary in the same mouse). One way to sort this out is by flow cytometry, other way is to do this by TCRa-/- and TCRb-/- and other way is to infect WT mice and doing a CD4+ t cell depletion before secondary infection.

We thank the Reviewer for this fully pertinent comment. Unfortunately and as mentioned above we are not able to consider additional investigations to specifically address this question. However, we agree that the interpretation of data in Figure 1 could have been subtler. We thus performed several important modifications in the title, introduction, results and discussion. We notably use "TCR-mediated immunity" rather than "adaptive immunity" when considering results obtained from $Rag2^{-/-}$ mice and we modified the title: "Th1 and Th17 immune responses act complementarily to optimally control superficial dermatophytosis". We also added supplementary data on infiltrating PMNs in skin of infected WT and $Rag2^{-/-}$ mice (Suppl. Figure S1a, b and c) that allow to highlight the PMNs innate cutaneous infiltration (independent of γδ T-cells).

These additional data were implemented as follows.

- In Results: p6 […] In both strains, the cell infiltration was composed of both monomorphonuclear and polymorphonuclear cells. Myeloperoxidase (MPO) activity assays, qRT-PCR evaluation of neutrophilic granule protein levels and direct counting on stained skin sections showed a significant skin infiltration of polymorphonuclear neutrophil (PMN)s at days 3 and 7 PI in both

strains of mice, which was significantly higher for the WT mice than for the Rag2$^{-/-}$ ones. At days 14 and 21 PI, WT mice had no more observable infiltrating PMNs, whereas skin of Rag2$^{-/-}$ mice still contained large amount of PMNs (Suppl. Figure S1a, b and c). […]

- In Materials and Methods: See Supplementary Materials and Methods for the histological procedure, evaluation of skin PMNs infiltration, evaluation of fungal load by CFU counting, single-cell preparation from LNs, *ex vivo* lymphoproliferative assay and cytokine production determination of cultured total skin-draining LNs cells, $CD4^+$ T-cell isolation and culture, ELISA, and qRT-PCR.
- In Figure 1 legend:

  […] These sections, stained with periodic acid-Schiff and counterstained with hematoxylin, show the fungal colonization of infected mice (yellow arrows), the dermal and perifollicular mononuclear (composed of both monomorphonuclear and polymorphonuclear cells) cell infiltration (black arrows), and the severe increase in the epidermis thickness. […]
- In Supplementary Materials and Methods
  - Evaluation of skin PMN infiltration

    The extent of PMN infiltration in the skin was evaluated by assaying myeloperoxidase (MPO) activity, qRT-PCR evaluation of neutrophilic granule protein levels (see qRT-PCR below) and direct counting on stained (hematoxylin/eosin) skin sections. For this last procedure, PMNs identified in micrographs by their distinctive nuclear morphology in three 40X fields per skin section were manually counted by two blinded observers.
  - Myeloperoxidase (MPO) activity measurement

    After thawing, the skin samples (0.1 g of tissue in 1.9 ml of buffer) were homogenized using the TissueLyser system (Qiagen Retsch GmbH, Hannover, Germany) in a pH 4.7 buffer 1 (0.1 M NaCl, 0.02 M Na_2PO_4, 0.015 M Na_2EDTA) (all from Sigma, St Louis, USA), centrifuged at 12000 *g*, at 4°C for 10 minutes. The pellets were resuspended in 100 µl of buffer 1 + 750 µl of a 0.2% NaCl solution + 750 µl of a NaCl 1.6% and glucose 5% solution and thereafter were again homogenized. After further centrifugation (12000 *g* at 4°C for 15 minutes), the pellets were resuspended in 1 ml of a 0.05 M Na_3PO_4 buffer (pH 5.4) containing 0.5% hexadecyl-trimethylammonium bromide (HTAB; Sigma, St. Louis, USA) and re-homogenized. Samples were then transferred into 1.5-ml microtubes, submitted to three freeze-thaw cycles using liquid nitrogen and then centrifuged at 12000 *g*, at 4°C for 15 minutes to perform the assay. The assay employed 25 µl of 3.4-5.6-tetramethylbenzidine (TMB; Sigma, St. Louis, USA), dissolved in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) at a final concentration of 1.6 mM, 100 µl of H_2O_2, dissolved in phosphate buffer (pH 5.4) containing HTAB at a final concentration of 0.002% vol/vol and 25 µl of sample. The reaction was started at 37°C for 5 minutes in a 96-well microplate by adding the supernatant and the TMB solution. After that, the H_2O_2

solution was added and the microplate was incubated at 37°C for 5 minutes. The reaction was stopped by adding 100 µl of 1 M H_2SO_4 and quantified at 450 nm in a spectrophotometer (Emax; Molecular Devices, Sunnyvale, USA). Results were expressed as absorbance per gram of skin.

- In Supplementary Figure Legends

  Supplementary Figure S1. **Comparative infiltration of PMNs in the skin of *T. benhamiae* infected WT and Rag2$^{-/-}$mice.**

  Rag2$^{-/-}$ mice and WT littermates were subjected to primary and secondary infections with *T. benhamiae*, with the secondary inoculation being performed 63 days after the first one. Inoculated skin samples were collected at days 3, 7, 14, and 21 PI and PMNs infiltration in the skin was evaluated by (**a**) qRT-PCR evaluation of neutrophilic granule protein (Ngp) levels (b) assaying myeloperoxidase (MPO) activity, and (**c**) direct counting on stained (hematoxylin/eosin) skin sections. Data are means ± standard deviations (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

In the objective of bringing out that Rag2$^{-/-}$ mice lack αβ and γδ T-cells and highlighting the contribution of the innate immune response in host defense against dermatophytes, the second paragraph of the discussion was modified as follows. p11 […] Using this model, we first showed that TCR-mediated immunity is critical for the optimal control of acute dermatophytosis as Rag2$^{-/-}$ mice presented long lasting infection with higher clinical scores and fungal loads than their WT counterparts. TCR-mediated immunity is achieved by adaptive immune cells but also by innate cells as γδ T-cells. It is well known that CD4+ T-cells are a crucial component of the immune defense against dermatophytes (Heinen et al., 2017) and importance of adaptive immunity is also emphasized by the high incidence of severe or long lasting dermatophyte infections reported in patients with acquired immune deficiency syndrome (Costa et al., 2015; Gupta et al., 2000). However, a recent report showed that adaptive immunity is dispensable for dermatophyte clearance in a mouse model of deep dermatophytosis, a rare condition induced by the anthropophilic pathogen *Trichophyton rubrum* (Dahl and Grando, 1994; Nenoff et al., 2014). Although it is important to consider the differences in experimental design, this previous study and our observation that the fungal load was higher in Rag2$^{-/-}$ mice than in WT controls at day 3 PI but decreased throughout the *T. benhamiae* infection suggest that, even though the adaptive immune response plays a critical role in controlling the disease, the contribution of the innate immune system is also important in host defense against dermatophytes. In mammals, it is generally accepted that the main effector and regulatory mechanisms in the fight against fungal skin infections are (i) increased epidermal turnover, (ii) cytotoxic and phagocytic activity of PMNs and macrophages, as well as (iii) the production of antimicrobial peptides (AMP)s at the site of infection (Blanco and Garcia, 2008; Brasch et al., 2014; Brown, 2011; Dahl, 1994; Lilic, 2012; Trzeciak-Ryczek et al., 2015; Verma et al., 2014; Wagner and Sohnle, 1995). These three defense mechanisms are induced in dermatophytosis and have been associated with dermatophyte

clearance (Heinen et al., 2017). It is also important to mention that during a superficial cutaneous fungal infection, all of these mechanisms can be triggered in a completely innate way. However, if they are insufficient to fight the infection, they will then be amplified by the cell-mediated immunity of the adaptive pathway and especially the Th1 and Th17 pathways. The main effectors cytokines of Th17 cells are IL-17 and IL-22. IL-17 promotes granulopoiesis and the production of PMN-chemoattractant chemokines. IL-17, either alone or in synergy with IL-22, regulates the expression of AMPs and IL-22 regulates the proliferation of keratinocytes (Boniface et al., 2005; Fujita, 2013; Gaffen, 2008; Kolls and Khader, 2010; Liang et al., 2006; Onishi and Gaffen, 2010). Th1 cell-secreted IFN-γ is involved in chemotaxis and is known to be essential for the optimal activation of phagocytic effector cell functions (Schroder et al., 2004). However, IL-17, IL-22 and IFN-γ are produced not only by Th1 and Th17 cells, but also by a number of other cell types including $CD8^+$ T-cells or innate cells such as γδ T-cells, lymphoid tissue inducer cells, innate lymphoid cells and natural killer cells (Annunziato et al., 2015; Ferretti et al., 2003; Gladiator et al., 2013; Isailovic et al., 2015; Montaldo et al., 2015). In addition, IL-17A was reported to be produced by PMNs (Ferretti et al., 2003; Taylor and Pearlman, 2016). Such innate cells can be skin resident or mobilized rapidly in response to infection and do not require prior antigen exposure to be activated. The lack of innate γδ T-cells in $Rag2^{-/-}$ mice could explain the increased fungal burden at day 3 PI compared to WT controls. Furthermore, synthesis of AMPs and attraction of PMNs can even be achieved without the intervention of this innate source of IL-17. For example, keratinocytes in culture are able to sense dermatophytes which leads to the induction of AMPs (Firat et al., 2014) and PMN-chemoattractant chemokines (Achterman et al., 2015; Shiraki et al., 2006; Tani et al., 2007). Thus this is not surprising to observe a PMN infiltration and a fungal clearance in $Rag2^{-/-}$ mice.

3. Minor points: a WT control in figure 5 would be nice.

A WT control was introduced.

4. Minor points: How does the clinical scoring relate to human disease and dermatophytosis. Do mice have similar phenotypes. Ie do Card9-/- mice have increased CFU (Lanternier et al., NEJM 2013)?

In immunocompetent humans, acute dermatophytic lesions are generally characterized by erythema, scaling and crusting on glabrous skin and additional alopecia in hairy areas (Brasch, 2009; Hay, 2007; Kick and Korting, 1998). However, the severity of the lesions varies considerably according to the host, the body site affected and the dermatophyte species (Achterman et al., 2015; Brasch, 2010; Degreef, 2008; Weitzman and Summerbell, 1995). In our mouse model, we observed all these lesions except alopecia that could not be evaluated mice having been shaved before inoculation.

Increased susceptibility to dermatophyte infections occurs in patients suffering from primary immunodeficiency (PID) where circulating IL-17 producing T-cells are decreased such as PID involving dectin-1 (Ferwerda et al., 2009) or CARD9 (Engelhardt and Grimbacher, 2012; Glocker et al., 2009). Similarly, PID related to CARD9 may be accompanied with an abnormally deep localization of dermatophytes (Alves de Medeiros et al., 2016; Drewniak et al., 2013; Glocker et al., 2009; Grumach et al., 2015; Lanternier et al., 2015; Lanternier et al., 2013; Wang and van de Veerdonk, 2016; Wang et al., 2014).

In the mice Yoshikawa and collaborators demonstrated that dectin-1 and dectin-2 are key elements of the immune response against *T. rubrum*, deficiency leading to compromised fungal clearance and impaired resolution of dermatophytosis in a model of deep infection (Yoshikawa et al., 2016). CARD9 is an adaptor acting downstream from receptors involved in fungal recognition, such as the mannose receptor, dectin-1, or dectin-2 (Bi et al., 2010; Drummond et al., 2011; Netea and Marodi, 2010). Thus based on the study of Yoshikawa and collaborators, an impaired dermatophyte clearance could be expected in CARD9$^{-/-}$ mice.

5. Minor points: it would be interesting to investigate the immune response of dermatophyte infection in humans by histology or flow cytometry but this is out of the scope of this manuscript.

We find this proposal exciting and pertinent for a further study. Several studies on dermatophyte infections in human showed the presence of IFN-γ in *tinea* lesions (Miyata et al., 1996; Slunt et al., 1996) and in culture of peripheral blood mononuclear cells (PBMC) challenged with trichophytin, a *Trichophyton* antigen (Koga et al., 1993; Koga et al., 2001b). More precisely IFN-γ-positive CD4+ T-cells were found to be enhanced in *tinea* lesions suggesting that the skin lesions caused by dermatophytes may be associated with a Th1 response (Koga et al., 2001a). However, the IL-17 mediated immunity was not investigated in any of the aforementioned studies. The IL-17-mediated immune response against dermatophytes in the skin of infected humans would be so a very interesting topic to be studied, but it was out of the scope of this manuscript.

6. Minor points: Would be interested in how this compares against a superficial yeast infection but this may also be out of the scope of this manuscript.

We also think that we could learn on anti-fungal skin immunity thanks to comparative studies between superficial yeast infections and dermatophytoses. This could be especially informative because dermatophytes, excepted geophilic species, are considered as obligatory parasites, which is not the case for opportunistic commensal pathogens such as *Candida* spp. and *Malassezia* spp. These differences should reflect the relevance of the different immunologic mechanisms that are involved in both types of infections, including in terms of tolerance and innate response.

Overall, this is a good manuscript that makes contributions to the investigative dermatology field. Refined and exact experimental approaches would significantly improve this manuscript for publication into the JID.

We hope that this revised version of our manuscript with new experimental data and a modified interpretation of several results taking into account the concepts developed by all the Reviewers will meet the Reviewer's expectations.

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Reviewer: **2**

Comments to the Author

Authors work on a very interesting but relatively unexplored aspect of cellular immunology. The mechanisms involved are of great interest to dermatology. Data are intriguing and fairly clean in terms of interpretation in some cases (Eg. Fig. 2), but authors do tend to make broad conclusions in others that are not warranted (Fig. 3). It is a bit disappointing to see that there are not more clear cut differences in the clinical scores in the various strains of mice, including even the double KO. The compensation data (hypothesis) looks good in vitro but the reality is that the double KO doesn't look much different than the IL17A KO. Perhaps an approach using RoRgt KO would be better. In any event, the model system they are using at least suggests an interesting mouse model by which investigation of the immunological responses to dermatophytes can be assessed. This in itself is an advance to the field.

The authors greatly appreciate the thoughtful *observations* and suggestions of Reviewer 2, which helped us to improve our manuscript. Please, see below our answers to Reviewer's queries and comments.

We think that the lack of clear cut differences in the clinical scores within the various strains of mice, including the double KO ones, could be explained by the fact that besides IL-17- and IFN-γ-mediated immunity (innate and adaptive immunity) there are a lot of other innate and innate-like mechanisms involved in the clearance of the dermatophyte. Indeed, keratinocytes possess PRRs which can recognize dermatophytes and can produce, during cutaneous infection, a wide range of cytokines, including chemokines that will attract phagocytic cells, as well as antimicrobial peptide (AMP)s and factors inducing their own proliferation. Keratinocytes therefore play a role in both the detection of the fungus and in the innate establishment of three main effector mechanisms of the fight against dermatophytes (see below). Nevertheless, these effector mechanisms triggered by keratinocytes can occur completely innately. As an example, *in vitro* cultured primary keratinocytes can detect dermatophytes leading to the induction of AMPs (Firat et al., 2014) and chemokines (Achterman et al., 2015; Shiraki et al., 2006; Tani et al., 2007). Thus, it is not surprising to observe a fungal decrease with inflammatory signs in all our infected deficient strains of mice, including in IL-17A and IFN-γ double-deficient mice.

We added supplementary data on infiltrating PMNs in skin of infected WT and $Rag2^{-/-}$ mice (Suppl. Figure S1a, b and c) that allow to highlight the PMNs innate cutaneous infiltration (independent of γδ T-cells). Additional data show that all the mentioned deficient strains were able to produce AMPs and attract PMNs at the site of the infection. Although this highlights the contribution

of IL-17- and IFN-γ- independent mechanisms, we chose not to publish these data in this manuscript, in the sole purpose not to complicate the message.

These supplementary data were implemented as follows.

- In Results: p7 […] In both strains, the cell infiltration was composed of both monomorphonuclear and polymorphonuclear cells. Myeloperoxidase (MPO) activity assays, qRT-PCR evaluation of neutrophilic granule protein levels and direct counting on stained skin sections showed a significant skin infiltration of polymorphonuclear neutrophil (PMN)s at days 3 and 7 PI in both strains of mice, which was significantly higher for the WT mice than for the Rag2$^{-/-}$ ones. At days 14 and 21 PI, WT mice had no more observable infiltrating PMNs, whereas skin of Rag2$^{-/-}$ mice still contained large amount of PMNs (Suppl. Figure S1a, b and c). […]
- In Materials and Methods: See Supplementary Materials and Methods for the histological procedure, evaluation of skin PMNs infiltration, evaluation of fungal load by CFU counting, single-cell preparation from LNs, *ex vivo* lymphoproliferative assay and cytokine production determination of cultured total skin-draining LNs cells, CD4^{+} T-cell isolation and culture, ELISA, and qRT-PCR.
- In Figure 1 legend:

  […] These sections, stained with periodic acid-Schiff and counterstained with hematoxylin, show the fungal colonization of infected mice (yellow arrows), the dermal and perifollicular mononuclear (composed of both monomorphonuclear and polymorphonuclear cells) cell infiltration (black arrows), and the severe increase in the epidermis thickness. […]
- In Supplementary Materials and Methods
  - Evaluation of skin PMN infiltration

    The extent of PMN infiltration in the skin was evaluated by assaying myeloperoxidase (MPO) activity, qRT-PCR evaluation of neutrophilic granule protein levels (see qRT-PCR below) and direct counting on stained (hematoxylin/eosin) skin sections. For this last procedure, PMNs identified in micrographs by their distinctive nuclear morphology in three 40X fields per skin section were manually counted by two blinded observers.
  - Myeloperoxidase (MPO) activity measurement

    After thawing, the skin samples (0.1 g of tissue in 1.9 ml of buffer) were homogenized using the TissueLyser system (Qiagen Retsch GmbH, Hannover, Germany) in a pH 4.7 buffer 1 (0.1 M NaCl, 0.02 M Na_2PO_4, 0.015 M Na_2EDTA) (all from Sigma, St Louis, USA), centrifuged at 12000 *g*, at 4°C for 10 minutes. The pellets were resuspended in 100 µl of buffer 1 + 750 µl of a 0.2% NaCl solution + 750 µl of a NaCl 1.6% and glucose 5% solution and thereafter were again homogenized. After further centrifugation (12000 *g* at 4°C for 15 minutes), the pellets were resuspended in 1 ml of a 0.05 M Na_3PO_4 buffer (pH 5.4) containing 0.5% hexadecyl-trimethylammonium bromide (HTAB; Sigma, St. Louis,

USA) and re-homogenized. Samples were then transferred into 1.5-ml microtubes, submitted to three freeze-thaw cycles using liquid nitrogen and then centrifuged at 12000 *g*, at 4°C for 15 minutes to perform the assay. The assay employed 25 µl of 3.4-5.6-tetramethylbenzidine (TMB; Sigma, St. Louis, USA), dissolved in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) at a final concentration of 1.6 mM, 100 µl of H_2O_2, dissolved in phosphate buffer (pH 5.4) containing HTAB at a final concentration of 0.002% vol/vol and 25 µl of sample. The reaction was started at 37°C for 5 minutes in a 96-well microplate by adding the supernatant and the TMB solution. After that, the H_2O_2 solution was added and the microplate was incubated at 37°C for 5 minutes. The reaction was stopped by adding 100 µl of 1 M H_2SO_4 and quantified at 450 nm in a spectrophotometer (Emax; Molecular Devices, Sunnyvale, USA). Results were expressed as absorbance per gram of skin.

- In Supplementary Figure Legends:

  Supplementary Figure S1. **Comparative infiltration of PMNs in the skin of *T. benhamiae* infected WT and Rag2$^{-/-}$ mice.**

  Rag2$^{-/-}$ mice and WT littermates were subjected to primary and secondary infections with *T. benhamiae*, with the secondary inoculation being performed 63 days after the first one. Inoculated skin samples were collected at days 3, 7, 14, and 21 PI and PMNs infiltration in the skin was evaluated by (**a**) qRT-PCR evaluation of neutrophilic granule protein (Ngp) levels (b) assaying myeloperoxidase (MPO) activity, and (**c**) direct counting on stained (hematoxylin/eosin) skin sections. Data are means ± standard deviations (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

In the objective of bringing out that Rag2$^{-/-}$ mice lack αβ and γδ T-cells and highlighting the contribution of the innate immune response in host defense against dermatophytes, the second paragraph of the discussion was modified as follows: p11 […] Using this model, we first showed that TCR-mediated immunity is critical for the optimal control of acute dermatophytosis as Rag2$^{-/-}$ mice presented long lasting infection with higher clinical scores and fungal loads than their WT counterparts. TCR-mediated immunity is achieved by adaptive immune cells but also by innate cells as γδ T-cells. It is well known that CD4+ T-cells are a crucial component of the immune defense against dermatophytes (Heinen et al., 2017) and importance of adaptive immunity is also emphasized by the high incidence of severe or long lasting dermatophyte infections reported in patients with acquired immune deficiency syndrome (Costa et al., 2015; Gupta et al., 2000). However, a recent report showed that adaptive immunity is dispensable for dermatophyte clearance in a mouse model of deep dermatophytosis, a rare condition induced by the anthropophilic pathogen *Trichophyton rubrum* (Dahl and Grando, 1994; Nenoff et al., 2014). Although it is important to consider the differences in

experimental design, this previous study and our observation that the fungal load was higher in Rag2$^{-/-}$ mice than in WT controls at day 3 PI but decreased throughout the *T. benhamiae* infection suggest that, even though the adaptive immune response plays a critical role in controlling the disease, the contribution of the innate immune system is also important in host defense against dermatophytes. In mammals, it is generally accepted that the main effector and regulatory mechanisms in the fight against fungal skin infections are (i) increased epidermal turnover, (ii) cytotoxic and phagocytic activity of PMNs and macrophages, as well as (iii) the production of antimicrobial peptides (AMP)s at the site of infection (Blanco and Garcia, 2008; Brasch et al., 2014; Brown, 2011; Dahl, 1994; Lilic, 2012; Trzeciak-Ryczek et al., 2015; Verma et al., 2014; Wagner and Sohnle, 1995). These three defense mechanisms are induced in dermatophytosis and have been associated with dermatophyte clearance (Heinen et al., 2017). It is also important to mention that during a superficial cutaneous fungal infection, all of these mechanisms can be triggered in a completely innate way. However, if they are insufficient to fight the infection, they will then be amplified by the cell-mediated immunity of the adaptive pathway and especially the Th1 and Th17 pathways. The main effectors cytokines of Th17 cells are IL-17 and IL-22. IL-17 promotes granulopoiesis and the production of PMN-chemoattractant chemokines. IL-17, either alone or in synergy with IL-22, regulates the expression of AMPs and IL-22 regulates the proliferation of keratinocytes (Boniface et al., 2005; Fujita, 2013; Gaffen, 2008; Kolls and Khader, 2010; Liang et al., 2006; Onishi and Gaffen, 2010). Th1 cell-secreted IFN-γ is involved in chemotaxis and is known to be essential for the optimal activation of phagocytic effector cell functions (Schroder et al., 2004). However, IL-17, IL-22 and IFN-γ are produced not only by Th1 and Th17 cells, but also by a number of other cell types including CD8^{+} T-cells or innate cells such as γδ T-cells, lymphoid tissue inducer cells, innate lymphoid cells and natural killer cells (Annunziato et al., 2015; Ferretti et al., 2003; Gladiator et al., 2013; Isailovic et al., 2015; Montaldo et al., 2015). In addition, IL-17A was reported to be produced by PMNs (Ferretti et al., 2003; Taylor and Pearlman, 2016). Such innate cells can be skin resident or mobilized rapidly in response to infection and do not require prior antigen exposure to be activated. The lack of innate γδ T-cells in Rag2$^{-/-}$ mice could explain the increased fungal burden at day 3 PI compared to WT controls. Furthermore, synthesis of AMPs and attraction of PMNs can even be achieved without the intervention of this innate source of IL-17. For example, keratinocytes in culture are able to sense dermatophytes which leads to the induction of AMPs (Firat et al., 2014) and PMN-chemoattractant chemokines (Achterman et al., 2015; Shiraki et al., 2006; Tani et al., 2007). Thus this is not surprising to observe a PMN infiltration and a fungal clearance in Rag2$^{-/-}$ mice.

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Fig. 1. The description of inflammatory cells in the H and E histology at least is sparse and characterized by "highly inflammatory". How about neutrophils? Staining for CD4 vs CD8 vs gamma delta T-cells, which have been described to produced IL-17 and 22 as well, should be performed for better characterization.

We totally agree with the Reviewer's comment. It would be very interesting to investigate the immune response against dermatophytes in the skin and to evaluate which cells are secreting IL-17 and IL-22 during an experimental infection. Unfortunately we have no more adequate samples to perform such experiments. Nevertheless, we added supplementary data on infiltrating PMNs in skin of infected WT and Rag2$^{-/-}$ mice (Suppl. Figure S1a, b and c) that allow to highlight the PMNs innate cutaneous infiltration (independent of γδ T-cells). Please, see previous comment.

After 21 days, does the infection eventually clear in the Rag2KO mice? Author should comment on this.

We added data on the evolution of clinical scores and fungal loads in *T. benhamiae* infected Rag2$^{-/-}$ mice (Suppl. Figure S2). As previously mentioned, we have already suggested that the fungal load decrease could be due to innate mechanisms (IL-17 dependent or independent).

These additional data were implemented as follows.

- In Results: p7 At the day 0 of secondary infection (day 63 post primary infection), Rag2$^{-/-}$ mice showed no more clinical signs and displayed undetectable fungal loads, as observed from the results of quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). However, fungal cultures from skin samples were deemed positive (4 ± 1.5 CFU/g) (Suppl. Figure S2a, b and c).
- In Supplementary Materials and Methods:
    - Supplementary Figure S2. Evolution of clinical scores and fungal loads in *T. benhamiae* infected Rag2$^{-/-}$ mice

      Rag2$^{-/-}$ mice were infected with *T. benhamiae* and at days 3, 7, 14, 21, 28, 35, 42, 49, 54 and 63 PI (**a**) clinical scores were evaluated and (**b**, **c**) fungal loads in the skin were determined by (**b**) CFU counting, and (**c**) qRT-PCR evaluation of chitin synthase (ChiS) levels. Data are means ± standard deviations (**$P < 0.01$, and ***$P < 0.001$).

Fig. 3. While clinical signs were exacerbated in the IFNgKO, the CFU scores are actually higher in the IL17AKO mice. Authors should comment on this. We

We have already commented on this in the discussion: The exacerbation of clinical signs in IFN-γ$^{-/-}$ mice, but not in IL-17A$^{/}$ IFN-γ^{DKO} mice, may be related to the associated overexpression of IL-17A in case of Th1 deficiency. The role of IL-17A in tissue damage is well documented. High IL-17A concentration may exacerbate inflammatory state, notably by increasing the recruitment of PMNs (Takagi et al., 2017; Zelante et al., 2007). Furthermore, Th1 cells-produced IFN-γ may also act

indirectly by inhibiting Th17 expansion, thereby limiting the inflammatory exacerbation and tissue damage.

It is not clear to me that IL-17AKO handle the fungal infection early better than IFNgamma.

I don't actually agree with their assertion that IL17A acts more efficiently early in the process. The only data they have is that IL17AKO show no difference from WT in clinical score. If IL17A were needed at an early point in infection, then one would expect to see a far greater clinical score at early time points compared to WT. This is clearly not the case as they are equal. Regarding their assertion that IFNgamma being involved in late clearance, there is little evidence that it is different from IL17A since the CFU data shows little difference between the IL-17A and IFNgKOs at day 14 and certainly both clear by day 21.

We perfectly agree with the Reviewer and we therefore modified the manuscript as follows:

In Abstract: we deleted the sentence: with IL-17A acting more efficiently during the early fungal clearance and IFN-γ being more involved in the elimination of the persistent fungi and reduction of the clinical signs. And we added at the end of the paragraph: adaptive immunity is polarized to both Th1 and Th17 responses with the Th17 antifungal response acting on dermatophyte clearance and the Th1 one being involved in both fungal clearance and Th17-inflammation down-modulation.

- In Results:

  p10 These data suggest that while IL-17A and IFN-γ are individually dispensable, they both contribute to the optimal resolution of dermatophytosis, with IL-17A acting on dermatophyte clearance and IFN-γ being involved in both fungal clearance and reduction of clinical signs.

In Discussion: p17 Th17 antifungal response acted more efficiently on dermatophyte clearance, whereas Th1 response participated in both fungal clearance and Th17-inflammation down-modulation.

Furthermore, as already explained in the first response, the second paragraph of the discussion was modified to bring out the fact that, besides IL-17- and IFN-γ-mediated immunity (innate and adaptive immunity), there are a lot of other innate and innate-like mechanisms involved in the clearance of the dermatophyte. This could explain the lack of clear cut differences in the clinical scores within the various strains of mice

Note: authors are using a major cytokine (IFN gamma with broad effects) IFNy KO in one case and only one cytokine IL17AKO in the other. What about IL17F and other family members? There are certainly caveats in the interpretation and readers should be made aware of them.

We agree with the Reviewer. An approach using RORγt$^{-/-}$ or IL-17RA$^{-/-}$ mice would probably have been more relevant for evaluating the role of the IL-17 family.

We added in the discussion:

p16: We have to note that the potential role of IL-17F and other cytokines of the IL-17 family was not explored in this study. Although totally unknown in dermatophyte infection, the role of IL-17F seems to be redundant with the one of IL-17A in defense against *S. aureus* and *Citrobacter rodentium* (Ishigame et al., 2009), but not in cutaneous *Candida albicans* infection where IL-17A$^{-/-}$ mice were shown to be susceptible to the infection despite having normal levels of IL-17F (Kagami et al., 2010).

P. 9 line 18. "Despite compensatory mechanisms, both single IL-17A and IFN-γ deficiencies affect the resolution of T. benhamiae infection." This statement is too strong (see above comments). Consider revising.

We agree with Reviewer's comment and we therefore modified the manuscript as follows.

- In Results:

  p11 We have deleted the sentence "Despite compensatory mechanisms, both single IL-17A and IFN-γ deficiencies affect the resolution of *T. benhamiae* infection" and replaced it with: In order to then investigate whether IL-17A and IFN-γ have complementary effects during disease resolution, mice deficient for […]

Fig. 5. How do authors explain the large increase in CFU in the double KO but very low clinical score? They should do a stain for dermatophytes at that late time point in the double KO (and other individual KO) to provide more proof that there are indeed many more dermatophytes in that case.

The large increase in CFU accompanied by low clinical scores observed in the double KO could be explained by the total absence of IL-17A, which leads to elevated fungal loads as observed in single-deficient IL-17A$^{-/-}$ mice without the IL-17 dependent exacerbation of clinical signs observed in single-deficient IFN-γ mice due to the compensatory overexpression of IL-17A.

At each time-point, we assessed the fungal presence/loads in all considered strains of mice by CFU counting (Figure 1e, Figure 4d, and Figure 6d), qRT-PCR evaluation of chitin synthase (ChiS) levels

(Figure 1f, Figure 4e and Figure 6e) and direct evaluation on skin sections stained with periodic acid-Schiff (data shown only for day 7 PI, Figure 1c, Figure 4b and Figure 6b). We think that this last analysis was less sensitive and specific than the others. Indeed, for CFU counting and qRT-PCR evaluation of ChiS levels, the whole area of infected skin was analyzed, while for histological procedure only a dozen of 4-µm-thick sections/sample was observed. This does not represent a significant area of the infected skin. Furthermore, there were high variations between the different sections of a same skin sample.

Minor

p. 5. Line 44, also used in clinical scoring details. "Squamosis" is not a commonly used word. How does it differ from crusting? Do authors mean "hyperkeratosis" or "epidermal hyperplasia". Please clarify, but suggest a different more precise term. These terms need to be better defined in the Methodology since it affects clinical scoring.

We agree with the Reviewer and replaced 'squamosis' by 'scaling' throughout the manuscript. Squamosis is a word commonly used in veterinary medicine but scaling is a better synonym which is different from crusting. We do not think that hyperkeratosis or epidermal hyperplasia are accurate terms for clinical signs because they rather reflect histological features.

The scoring of experimental lesions was first established by Greenberg and collaborators in guinea pigs using several parameters such as the size of skin lesions and the severity of erythema, scale, crust and scar (Greenberg et al., 1976). Indeed, in guinea pig the common clinical signs associated with experimental dermatophytosis are alopecia, scaling and crusting (Chittasobhon and Smith, 1979; Koch and Rieth, 1958; Kraemer et al., 2013). Thereafter, these parameters were also employed with slight modifications by numerous scientists working with similar Guinea pig models of dermatophytosis (Cambier et al., 2015; Grumbt et al., 2011; Mignon et al., 1999; Staib et al., 2010; Vermout et al., 2004). Parameters were also adapted in a mouse model of dermatophytosis in our laboratory (Cambier et al., 2014). For this study, we adapted this last scoring system (Cambier et al., 2014) by abandoning the criterium "alopecia", especially because this symptom appears to be difficult to consider when inoculation is performed on a shaved area and because disease evolution is rapid.

We defined the terms in Supplementary materials and methods:

[...] scaling (visible accumulation of loose fragments of the horny layer (stratum corneum) of the skin) and crusting (accumulation of dried exudate, serum, pus, blood, cells, and thick adherent scales on the skin surface) [...]

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Reviewer: 3

Comments to the Author

The authors dissect the role of Rag-dependent cells and the role of IL-17 and IFN-gamma effector cytokines in a murine model of primary and secondary challenge with a dermatophyte. The data is presented in a clear manner and the experimental interpretation is appropriate. While the authors explore the role of IFN-gamma and IL-17 in this model, the cellular sources of the mediators are not identified and I feel that this a critical part of this study.

Authors would like to thank the Reviewer 3 for his/her positive remarks as well as for his constructive comments. Please, see below our answers to Reviewer's queries.

1. The authors should identify IFN-gamma and IL-17-producing cells in their epicutaneous infection model. Do ILCs represent a major source at the earliest time point (day 3) post-challenge, as has been reported in other models of superfical mycoses? Alternatively, gd T cells may play an important early role. The phentopye of Rag2-/- vs. Rag2-/-common gamma chain-/- would be instructive as well, if available.

We totally agree with this comment. IL-17 and IFN-γ are produced by a number of adaptive and innate immune cells (see below) and it could be very relevant to identify the producing cells in our model. Unfortunately, *f*or technical reasons, we cannot perform additional experiments to better characterize the cytokine response at the cellular level using flow cytometry and/or other deficient strains of mice.

Since the discovery of IL-17-producing γδ T-cells in mice in 2005 (Stark et al., 2005), immunologists made considerable progress in defining the role of these cells. This functional subset has been shown to play a protective role in host defense against microbes, such as *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Listeria monocytogenes* (Hamada et al., 2008; Meeks et al., 2009; Okamoto Yoshida et al., 2010; Umemura et al., 2007; Xu et al., 2010), *Escherichia coli* (Hamada et al., 2008; Meeks et al., 2009; Riol-Blanco et al., 2010; Xu et al., 2010) and *Candida albicans* (Conti et al., 2014; Dejima et al., 2011; Hirota et al., 2011). In these models of infection, γδ T-cells produce IL-17 at an early stage of the immune response, and this early production of IL-17 is required for polymorphonuclear neutrophil (PMN)s recruitment at the site of infection and the subsequent clearance of the pathogen.

Similarly, Gladiator and collaborators showed that innate lymphoid cell (ILC)3s are the major source of IL-17 in response to *C. albicans* and that, unlike T-cells, these cells are essential and sufficient for IL-17–mediated protective immunity against the fungus in the oral mucosa (Gladiator et al., 2013).

Although it would be very interesting to investigate, the role of these innate cells has not yet been studied in dermatophytosis. Unfortunately and as mentioned above, we are unable to perform additional investigations because of a lack of remaining adequate samples and funding.

However, in the objective of bringing out that Rag2$^{-/-}$ mice lack αβ and γδ T-cells and of highlighting the contribution of the innate immune response in host defense against dermatophytes we performed several important modifications in the title, introduction, results and discussion. We notably use "TCR-mediated immunity" rather than "adaptive immunity" when considering results obtained from Rag2$^{-/-}$ mice and we modified the title: "Th1 and Th17 immune responses act complementarily to optimally control superficial dermatophytosis". We also added supplementary data on infiltrating PMNs in skin of infected WT and Rag2$^{-/-}$ mice (Suppl. Figure S1a, b and c) that allow to highlight the PMNs innate cutaneous infiltration (independent of γδ T-cells).

We modified the manuscript as follows.

- In Results: p7 […] In both strains, the cell infiltration was composed of both monomorphonuclear and polymorphonuclear cells. Myeloperoxidase (MPO) activity assays, qRT-PCR evaluation of neutrophilic granule protein levels and direct counting on stained skin sections showed a significant skin infiltration of polymorphonuclear neutrophil (PMN)s at days 3 and 7 PI in both strains of mice, which was significantly higher for the WT mice than for the Rag2$^{-/-}$ ones. At days 14 and 21 PI, WT mice had no more observable infiltrating PMNs, whereas skin of Rag2$^{-/-}$ mice still contained large amount of PMNs (Suppl. Figure S1a, b and c). […]
- In Materials and Methods: See Supplementary Materials and Methods for the histological procedure, evaluation of skin PMNs infiltration, evaluation of fungal load by CFU counting, single-cell preparation from LNs, *ex vivo* lymphoproliferative assay and cytokine production determination of cultured total skin-draining LNs cells, CD4^{+} T-cell isolation and culture, ELISA, and qRT-PCR.
- In Figure 1 legend:

  […] These sections, stained with periodic acid-Schiff and counterstained with hematoxylin, show the fungal colonization of infected mice (yellow arrows), the dermal and perifollicular mononuclear (composed of both monomorphonuclear and polymorphonuclear cells) cell infiltration (black arrows), and the severe increase in the epidermis thickness. […]
- In Supplementary Materials and Methods
  - Evaluation of skin PMN infiltration

    The extent of PMN infiltration in the skin was evaluated by assaying myeloperoxidase (MPO) activity, qRT-PCR evaluation of neutrophilic granule protein levels (see qRT-PCR below) and direct counting on stained (hematoxylin/eosin) skin sections. For this last procedure, PMNs identified in micrographs by their distinctive nuclear morphology in three 40X fields per skin section were manually counted by two blinded observers.

- Myeloperoxidase (MPO) activity measurement

  After thawing, the skin samples (0.1 g of tissue in 1.9 ml of buffer) were homogenized using the TissueLyser system (Qiagen Retsch GmbH, Hannover, Germany) in a pH 4.7 buffer 1 (0.1 M NaCl, 0.02 M Na_2PO_4, 0.015 M Na_2EDTA) (all from Sigma, St Louis, USA), centrifuged at 12000 *g*, at 4°C for 10 minutes. The pellets were resuspended in 100 µl of buffer 1 + 750 µl of a 0.2% NaCl solution + 750 µl of a NaCl 1.6% and glucose 5% solution and thereafter were again homogenized. After further centrifugation (12000 *g* at 4°C for 15 minutes), the pellets were resuspended in 1 ml of a 0.05 M Na_3PO_4 buffer (pH 5.4) containing 0.5% hexadecyl-trimethylammonium bromide (HTAB; Sigma, St. Louis, USA) and re-homogenized. Samples were then transferred into 1.5-ml microtubes, submitted to three freeze-thaw cycles using liquid nitrogen and then centrifuged at 12000 *g*, at 4°C for 15 minutes to perform the assay. The assay employed 25 µl of 3.4-5.6-tetramethylbenzidine (TMB; Sigma, St. Louis, USA), dissolved in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) at a final concentration of 1.6 mM, 100 µl of H_2O_2, dissolved in phosphate buffer (pH 5.4) containing HTAB at a final concentration of 0.002% vol/vol and 25 µl of sample. The reaction was started at 37°C for 5 minutes in a 96-well microplate by adding the supernatant and the TMB solution. After that, the H_2O_2 solution was added and the microplate was incubated at 37°C for 5 minutes. The reaction was stopped by adding 100 µl of 1 M H_2SO_4 and quantified at 450 nm in a spectrophotometer (Emax; Molecular Devices, Sunnyvale, USA). The results were expressed as relative units to the respective uninfected controls.

- In Supplementary Figure Legends

  Supplementary Figure S1. **Comparative infiltration of PMNs in the skin of *T. benhamiae* infected WT and Rag2$^{-/-}$ mice.**

  Rag2$^{-/-}$ mice and WT littermates were subjected to primary and secondary infections with *T. benhamiae*, with the secondary inoculation being performed 63 days after the first one. Inoculated skin samples were collected at days 3, 7, 14, and 21 PI and PMNs infiltration in the skin was evaluated by (**a**) qRT-PCR evaluation of neutrophilic granule protein (Ngp) levels (b) assaying myeloperoxidase (MPO) activity, and (**c**) direct counting on stained (hematoxylin/eosin) skin sections. Data are means ± standard deviations (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

- In the discussion: p12 […] Using this model, we first showed that TCR-mediated immunity is critical for the optimal control of acute dermatophytosis as Rag2$^{-/-}$ mice presented long lasting infection with higher clinical scores and fungal loads than their WT counterparts. TCR-mediated immunity is achieved by adaptive immune cells but also by innate cells as γδ T-cells. It is well known that CD4+ T-cells are a crucial component of the immune defense against dermatophytes

(Heinen et al., 2017) and importance of adaptive immunity is also emphasized by the high incidence of severe or long lasting dermatophyte infections reported in patients with acquired immune deficiency syndrome (Costa et al., 2015; Gupta et al., 2000). However, a recent report showed that adaptive immunity is dispensable for dermatophyte clearance in a mouse model of deep dermatophytosis, a rare condition induced by the anthropophilic pathogen *Trichophyton rubrum* (Dahl and Grando, 1994; Nenoff et al., 2014). Although it is important to consider the differences in experimental design, this previous study and our observation that the fungal load was higher in Rag2$^{-/-}$ mice than in WT controls at day 3 PI but decreased throughout the *T. benhamiae* infection suggest that, even though the adaptive immune response plays a critical role in controlling the disease, the contribution of the innate immune system is also important in host defense against dermatophytes. In mammals, it is generally accepted that the main effector and regulatory mechanisms in the fight against fungal skin infections are (i) increased epidermal turnover, (ii) cytotoxic and phagocytic activity of PMNs and macrophages, as well as (iii) the production of antimicrobial peptides (AMP)s at the site of infection (Blanco and Garcia, 2008; Brasch et al., 2014; Brown, 2011; Dahl, 1994; Lilic, 2012; Trzeciak-Ryczek et al., 2015; Verma et al., 2014; Wagner and Sohnle, 1995). These three defense mechanisms are induced in dermatophytosis and have been associated with dermatophyte clearance (Heinen et al., 2017). It is also important to mention that during a superficial cutaneous fungal infection, all of these mechanisms can be triggered in a completely innate way. However, if they are insufficient to fight the infection, they will then be amplified by the cell-mediated immunity of the adaptive pathway and especially the Th1 and Th17 pathways. The main effectors cytokines of Th17 cells are IL-17 and IL-22. IL-17 promotes granulopoiesis and the production of PMN-chemoattractant chemokines. IL-17, either alone or in synergy with IL-22, regulates the expression of AMPs and IL-22 regulates the proliferation of keratinocytes (Boniface et al., 2005; Fujita, 2013; Gaffen, 2008; Kolls and Khader, 2010; Liang et al., 2006; Onishi and Gaffen, 2010). Th1 cell-secreted IFN-γ is involved in chemotaxis and is known to be essential for the optimal activation of phagocytic effector cell functions (Schroder et al., 2004). However, IL-17, IL-22 and IFN-γ are produced not only by Th1 and Th17 cells, but also by a number of other cell types including CD8^{+} T-cells or innate cells such as γδ T-cells, lymphoid tissue inducer cells, innate lymphoid cells and natural killer cells (Annunziato et al., 2015; Ferretti et al., 2003; Gladiator et al., 2013; Isailovic et al., 2015; Montaldo et al., 2015). In addition, IL-17A was reported to be produced by PMNs (Ferretti et al., 2003; Taylor and Pearlman, 2016). Such innate cells can be skin resident or mobilized rapidly in response to infection and do not require prior antigen exposure to be activated. The lack of innate γδ T-cells in Rag2$^{-/-}$ mice could explain the increased fungal burden at day 3 PI compared to WT controls. Furthermore, synthesis of AMPs and attraction of PMNs can even be achieved without the intervention of this innate source of IL-17. For example, keratinocytes in culture are able to sense dermatophytes which leads to the induction of AMPs

(Firat et al., 2014) and PMN-chemoattractant chemokines (Achterman et al., 2015; Shiraki et al., 2006; Tani et al., 2007). Thus this is not surprising to observe a PMN infiltration and a fungal clearance in Rag2$^{-/-}$ mice.

2. The authors should have the manuscript edited by a native English speaker.

The manuscript "Th1 and Th17 immune responses act complementarily to control superficial dermatophytosis" by Heinen Marie-Pierre and co-authors was edited by Elsevier Language Editing Services (see attached certificate).

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