Keratinocyte IL-36 receptor/MyD88 signaling is involved in *Malassezia*-induced IL-17-dependent skin inflammation

（ケラチノサイトのIL-36受容体/MyD88経路が
マラセチア誘導性IL-17依存性皮膚炎に関与する）

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ABSTRACT

Background: Among skin commensal fungi, lipophilic *Malassezia* species exist on nearly all human skin surfaces. They are associated with mild to severe inflammatory skin diseases ranging from dandruff to atopic dermatitis. The pathophysiology of *Malassezia*-associated skin diseases has not been well understood, partly because of a lack of appropriate animal models.

Objective: We investigated the mechanisms underlying *Malassezia*-induced skin inflammation using a novel murine model that physiologically recapitulates *Malassezia* skin infection.

Methods: Mice were epicutaneously inoculated with *Malassezia* yeasts without barrier dysfunction and with no lipid supplementation. They were then evaluated for skin inflammation by clinically counting skin disease scores and histopathologically measuring epidermal thickness, by assessing lesional fungal loads (*Malassezia* colony-forming units) and by quantitating expression of cytokines and antimicrobial peptides in lesions at the mRNA/protein levels.

Results: *Malassezia* induced skin inflammation by day 4 post-inoculation and induced epidermal thickening in wild-type mice. High fungal burdens in the cornified layer (day 2 post-inoculation) were decreased thereafter with complete clearance by day 7 post-inoculation. *Malassezia* induced production of type 17 and type 17-polarizing cytokines in addition to antimicrobial peptides in inflamed skin. Using IL-17-knockout mice or mice deficient in specific immune cell types, we showed that *Malassezia*-induced skin inflammation was IL-17-dependent and required significant participation from group 3 innate lymphoid cells. Moreover, IL-17-dependent skin inflammation was mediated by keratinocyte MyD88 signaling in which the IL-36 receptor, but not the IL-1 receptor,

contributed as an upstream molecule.

Conclusion: This skin infection model showed that *Malassezia*-induced IL-17-dependent skin inflammation is mediated by the IL-36 receptor/MyD88 signaling.

Key messages

- *Malassezia*, commensal fungi of the skin, induce IL-17-dependent skin inflammation in which group 3 innate lymphoid cells play a major role.
- *Malassezia*-induced skin inflammation is mediated by keratinocyte MyD88 signaling in which the IL-36 receptor, but not the IL-1 receptor, is involved.

Capsule summary

The commensal fungi, *Malassezia*, exist on all human skin, where they interact with skin cells immunologically affecting health and disease. Here, the underlying response to the fungi was uncovered using a novel animal model.

Key words: Fungus, *Malassezia*, skin, infection, inflammation, keratinocytes, IL-36, MyD88, IL-17, group 3 innate lymphoid cells

Abbreviations:

CFU: Colony-forming unit, DETCs: dendritic epidermal T cells, ILCs: innate lymphoid cells, ILC3s: group 3 innate lymphoid cells, IL: interleukin, IL-1R: interleukin-1 receptor, H&E: hematoxylin and eosin, mAb: monoclonal antibody, PAS: periodic acid-Schiff , p.i.: post-inoculation, *spp.*: species, TLR: Toll-like receptor, WT: wild-type

INTRODUCTION

Malassezia yeasts are human commensal fungi, found most abundantly in the skin and less abundantly in the gut, that are associated with health and disease.[1-3] *Malassezia* species (*spp.*) have been implicated in the pathogenesis of a number of skin diseases such as dandruff, seborrheic dermatitis, pityriasis versicolor, *Malassezia* folliculitis, psoriasis and atopic dermatitis.[4-6] In the gut, they have more recently been reported to exacerbate colitis in Crohn's disease[7] and even to promote oncogenesis of pancreatic cancer.[8] In healthy individuals, *Malassezia* yeasts asymptomatically colonize nearly all skin surfaces with few exceptions (e.g., the toe-web space) and predominate in the skin fungal microbiome, reflecting their benign commensal roles.[1, 2] In contrast to *Candida* yeasts, commensal fungi that are less abundantly found at the cutaneous and mucosal surfaces of healthy individuals and are quite often involved in superficial and invasive infections in patients with various immunocompromising conditions,[9, 10] *Malassezia* yeasts are mainly associated with the skin diseases mentioned above and seldom cause systemic infections even in immuno-suppressed or immuno-deficient conditions.[11] The pathophysiology of *Malassezia*-associated skin diseases is poorly understood, in part due to the lack of known inborn errors of immunity showing clear causative links between *Malassezia* and disease development in humans and in part due to a lack of appropriate animal models to study *in vivo* host-pathogen interactions. Thus, our knowledge of the interactions between *Malassezia* yeasts and the human host largely relies on *in vitro* experiments.[12]

Sparber et al., however, recently established an infection model involving application of *Malassezia* yeasts onto the ear skin of mice.[6] They provided insight into host-*Malassezia* interactions in the skin by demonstrating that type 17 immunity

controls fungal colonization and also drives *Malassezia*-associated inflammation in barrier-disrupted skin, mimicking atopic dermatitis. In their model, olive oil-suspended *Malassezia* yeasts were topically applied onto the ear skin without any dressing. Olive oil provided lipid supplementation to enable *Malassezia* yeasts to more efficiently colonize the ear skin because most *Malassezia spp.* are lipophilic and require external lipid sources for growth.[11] In addition, *Malassezia* infection of ear skin that was barrier-disrupted by tape stripping induced more severe skin inflammation than infection without tape stripping. To more closely recapitulate superficial *Malassezia* skin infection, we established a novel murine *Malassezia* skin infection model without skin barrier disruption and with no external lipid supplementation. Using this model, we found that *Malassezia* yeasts induce interleukin (IL)-17-dependent skin inflammation in which the IL-36 receptor/MyD88 signaling in keratinocytes is involved.

METHODS

Animals

Wild-type (WT) mice on a C57BL/6J background were purchased from Japan CLEA (Tokyo, Japan). *Il17a*$^{-/-}$*f*$^{/-}$,[13, 14] *Rorc*$^{-/-}$,[15, 16] *Rag2*$^{-/-}$,[17] *Myd88*$^{-/-}$,[18] *Tlr2*$^{-/-}$*4*$^{-/-}$,[19, 20] and *Il1r*$^{-/-}$ [21] mice on C57BL/6 backgrounds were previously reported. *K14-Cre* mice were purchased from the Jackson Laboratory. *Myd88*$^{fl/fl}$ mice on a C57BL/6 background were a gift from Dr. Xiaoxia Li (Cleveland Clinic, Cleveland, Ohio).[22] The generation of *K14-Cre-Myd88*$^{fl/fl}$ (*Myd88*$^{\Delta ker}$) mice with a conditional deletion of MyD88 in keratinocytes has been previously described.[23]All mouse strains were bred and maintained under specific pathogen-free (SPF) conditions and were used in experiments at 8 to 12 weeks of age. Female mice were used in all experiments. Phosphate-buffered saline (PBS)-treated and *Malassezia*-infected mice were kept separately to avoid cross-contamination. No *Malassezia spp.* was detected by culture in our colony of SPF mice prior to infection. All animal studies were performed according to protocols approved by the Chiba University Review Board for Animal Care and in strict accordance with the Regulations for Animal Experimentation at Chiba University.

M. furfur culture

Malassezia furfur (IFM 40081) was kindly provided by the Medical Mycology Research Center, Chiba University. *M. furfur* was incubated for 4–5 days at 30°C with 180 rpm shaking in liquid-modified Leeming and Notman agar [LNA GT(10); for 1 L liquid medium 10 g peptone (Thermo Fisher Scientific, Waltham, Mass), 5 g glucose (Sigma-Aldrich, St Louis, Mo), 0.1 g yeast extract (Thermo Fisher Scientific), 8 g oxgall (Becton, Dickinson and Company, Franklin Lakes, NJ), 10 mL glycerol (Wako

Pure Chemical Industries, Osaka, Japan), 0.5 g glycerol monostearate (Santa Cruz Biotechnology, Santa Cruz, Calif), and 5 mL Tween 60 (Sigma-Aldrich); 12 g of agar (Wako Pure Chemical Industries) was added for plates].[24] To determine the number of colony forming units (CFUs), samples were plated on LNA GT(10) agar plates supplemented with 0.05% chloramphenicol (Wako Pure Chemical Industries) and incubated for 4–5 days at 30°C.

M. furfur infection model

The dorsal skin of mice was shaved using an electric clipper, and then depilatory cream (Veet® In Shower Hair Removal Cream; Reckitt Benckiser, Slough, United Kingdom) was gently applied for further hair removal. Prior to fungal inoculation, the skin was histopathologically orthokeratotic, reflecting little, if any, epidermal barrier dysfunction. Cultured *M. furfur* cells were washed and resuspended in PBS and diluted to an optical density at 600 nm (OD_{600}) of 20 immediately before inoculation of mouse skin. One hundred microliters of *M. furfur* cell suspension (corresponding to approximately 5×10^6 CFU) was placed on a patch of sterile gauze (1×1 cm), which was then attached to the shaved skin with transparent bioocclusive dressing (3M, Maplewood, Minn). Each mouse was exposed to *M. furfur* for 2, 4 or 7 days through the patch. The skin disease score was assessed by modifying the scoring system described previously.[23, 25] In brief, the skin disease score was the sum of individual scores for erythema, scaling, erosion, and thickness, graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe). Mice were then sacrificed, and skin samples were used for histology, CFU enumeration, RNA extraction and cytokine quantitation.

Antibody treatment of mice

To deplete innate lymphoid cells (ILCs), mice were treated with an anti-CD90 monoclonal antibody (mAb) (200 μg; clone: 30H12; Bio X Cell, West Lebanon, NH) or a control mAb (200 μg; clone: LTF-2; Bio X Cell) intraperitoneally and subcutaneously on days -4, -2, 0, and 2 of *M. furfur* infection. To neutralize the IL-36 receptor (IL-36R), mice were injected intradermally on day 0 and intraperitoneally on days 1 and 3 with an anti-IL-36R mAb (50 μg; clone: M616; Amgen, Thousand Oaks, Calif) or a control mAb (50 μg; clone: 2A3; Bio X Cell).

Histology

Skin tissue was formalin fixed, paraffin embedded, and sectioned for hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining. For each mouse, 15 epidermal thickness measurements from images taken at 100× magnification using a BX41 microscope equipped with DP20 camera (Olympus, Tokyo, Japan) were averaged using ImageJ software version 1.52 (National Institutes of Health, Bethesda, Md) to determine the epidermal thickness.

Isolation of murine primary keratinocytes and *in vitro* stimulation

Primary keratinocytes were isolated from WT mice as previously described.[23] Primary keratinocytes were cultured in proliferation medium (CnT-PR; CELLnTEC, Bern Switzerland) and passaged once. When cells reached confluency, terminal differentiation of keratinocytes was induced by switching the culture medium to differentiation medium (CnT-PR-D; CELLnTEC) supplemented with 1.2 mM $CaCl_2$. Simultaneously, primary keratinocytes were stimulated with live *M. furfur* at a

predetermined multiplicity of infection of 30 or mock-stimulated for 24 h at 37°C under a humidified atmosphere containing 5% CO_2. Following stimulation, cell lysates were collected for RNA extraction and supernatants were collected for cytotoxicity assays.

RNA extraction from mouse skin tissue and primary keratinocytes

Total RNA was extracted from homogenized skin tissue using an EZNA® Total RNA Kit I (Omega bio-tek, Norcross, Ga) and from primary keratinocytes using ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacturers' instructions. Total RNA was used to synthesize cDNA using PrimeScript™ RT Master Mix (Perfect Real Time) (Takara Bio, Shiga, Japan) according to the manufacturer's instructions.

Real-time quantitative reverse transcription PCR (qPCR)

qPCR was performed using TB Green® *Premix Ex Taq*™ II (Takara Bio) and a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, Calif). Expression levels were normalized to the expression of *Gapdh*. The primer sequences used were as follows: *Gapdh*, 5'-TGTGTCCGTCGTGGATCTGA-3' and 5'-TTGCTGTTGAAGTCGCAGGAG-3'; *Il17a*, 5'- CAGGGAGAGCTTCATCTGTGT-3' and 5'-GCTGAGCTTTGAGGGATGAT-3'; *Il17f*, 5'CTGTTGATGTTGGGACTTG-3' and 5'-GTTCATGGTGCTGTCTTCCTG-3'; *Il17c*, 5'- CTGGAAGCTGACACTCACG -3' and 5'- GGTAGCGGTTCTCATCTGTG -3'; *Il22*, 5'-TGACGACCAGAACATCCAGA-3' and 5'-AGCTTCTTCTCGCTCAGACG-3'; *Il6*, 5'-CCAGAGATACAAAGAAATGATGG-3' and 5'-ACTCCAGAAGACCAGAGGAAAT-3'; *Il1b*, 5'-TCTTCTTTGGGTATTGCTTGG-3' and 5'-TGTAATGAAAGACGGCACACC-3'; *Defb3*, 5'-

TCTGTTTGCATTTCTCCTGGTG-3’ and 5’-TCTGACGAGTGTTGCCAATG-3’; *S100a9*, 5’-ACTCTTTAGCCTTGAAGAGCAAG-3’ and 5’-TTCTTGCTCAGGGTGTCAGG-3’; *Il36a*, 5’-GTTCGTCTCAAGAGTGTCCAGATAT-3’ and 5’-ACTCCAGAAGACCAGAGGAAAT-3’; *Il36b*, 5’-CCATGTTGGATTTACTTCTCAGACT-3’ and 5’-ACAAAAAGCCTTTCTGTTCTATCAT-3’; *Il36g*, 5’-AGAGTAACCCCAGTCAGCGTG-3’ and 5’-AGGGTGGTGGTACAAATCCAA-3’.

Cytometric bead assay

Levels of IL-17A and IL-17F proteins in skin tissue homogenates were measured using BD CBA Flex Sets according to the manufacturer’s instructions (BD Biosciences, San Jose, CA). The results were analyzed using FCAP Array version 3.0 software (BD Bioscience).

Cytotoxicity assay

Cytotoxicity was measured using a lactate dehydrogenase release assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay; Promega, Madison, Wis) according to the manufacturer’s instructions.

Statistical analyses

Statistical analyses were performed using GraphPad Prism software version 7.0e (Graphpad Software, San Diego, Calif). Differences between two groups were assessed using the Mann–Whitney U test. Differences among multiple groups were assessed

using one-way analysis of variance (ANOVA) (parametric) or the Kruskal–Wallis test (non-parametric) with post-hoc testing. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Epicutaneous infection of mouse skin with *M. furfur*

To investigate how *Malassezia* yeasts interact with the skin *in vivo* under near-physiological conditions (both for the skin and for the pathogen), we established a new infection model in which the dorsal skin of mice was inoculated with *M. furfur* without disruption of skin barriers and with no external lipid supplementation. On day 2 post-inoculation (p.i.), WT mice (C57BL/6J background) showed mild skin disease with no significant epidermal thickening. The mice showed more robust skin disease with significant epidermal thickening and excessive cornification (i.e., hyperkeratosis) on day 4 p.i., and thereafter on day 7 p.i. showed slightly improved skin pathology. By contrast, mock-infected control mice showed normal skin with no epidermal thickening and hyperkeratosis (Fig 1, *A–C*). Interestingly, the epidermal and dermal inflammatory infiltrates were mild even on day 4 p.i. (Fig 1, *A*). *Malassezia* yeasts were localized in the outermost layers of the thickened cornified layers as shown in the inset of Fig 1, *A*. Their localization was consistent with previously reported observations.[6] The fungal burden decreased over the observation period and was eventually undetectable on day 7 p.i., as assessed by counting *M. furfur* CFUs in the skin (Fig 1, *D*).

M. furfur induce type 17 inflammation

Considering that IL-17 and related cytokines were involved in fungal immunity,[26, 27] we examined their expression in the tissues in *M. furfur*-induced skin inflammation. Type 17 cytokines (i.e., IL-17A, IL-17F, IL-17C, and IL-22), type 17-polarizing cytokines (i.e., IL-6 and IL-1β) and antimicrobial peptides induced by IL-17 (i.e., β-defensin 3 and S100A9) were all up-regulated in lesional skin of mice inoculated with

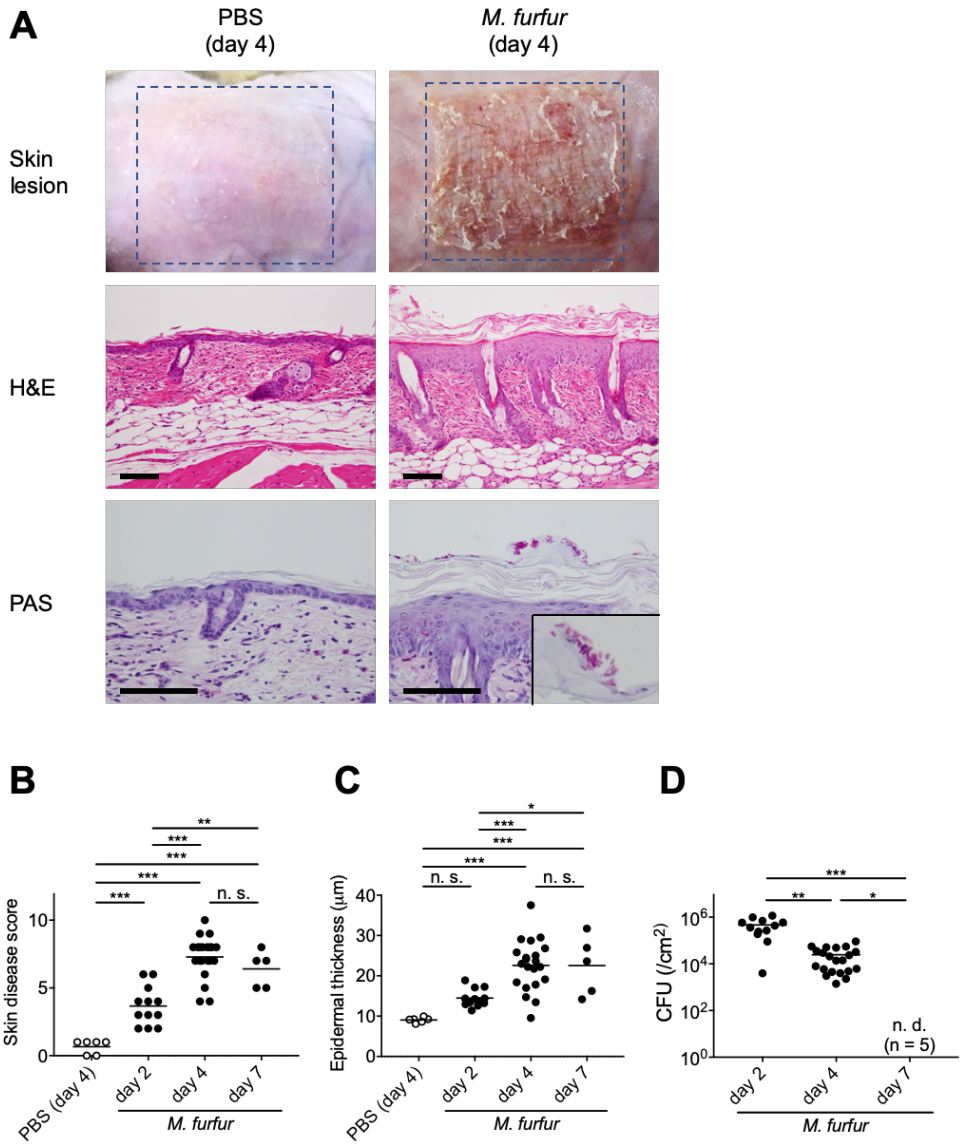

Figure 1. The mouse model of epicutaneous *M. furfur* infection. A, C57BL/6J mice were epicutaneously inoculated with *M. furfur* for 4 days. Mice treated with vehicle (PBS) alone are shown for comparison. Representative macroscopic images of skin lesions and H&E- or PAS-stained skin sections are shown. Inset shows high-power image of colonized *Malassezia* yeasts. Scale bars, 50 μm. **B–D,** Mice were epicutaneously inoculated with *M. furfur* for 2, 4, or 7 days or treated with PBS for 4 days for comparison (Fig 1, *B* and *C*). Skin disease scores (Fig 1, *B*) and epidermal thickness (Fig 1, *C*) of mice on the indicated days are shown. *M. furfur* CFUs on days 2, 4, and 7 p.i. are shown in Fig 1, *D*. Data represent combined results from four independent experiments (Fig 1, *B–D*). Each dot represents an individual mouse. n.s., not significant; n.d., not detected; *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with Tukey's multiple comparison test (Fig 1, *B* and *C*) or Kruskal–Wallis test with Dunn's multiple comparison test (Fig 1, *D*).

M. furfur compared with the skin of mock-infected animals (Fig 2, *A–C*). We also confirmed that production of IL-17A and IL-17F were up-regulated upon *M. furfur* infection (Fig 2, *D*). These results were consistent with previous data using a different model in which *Malassezia* yeasts were applied to the dorsal ear skin with or without skin barrier disruption.[6]

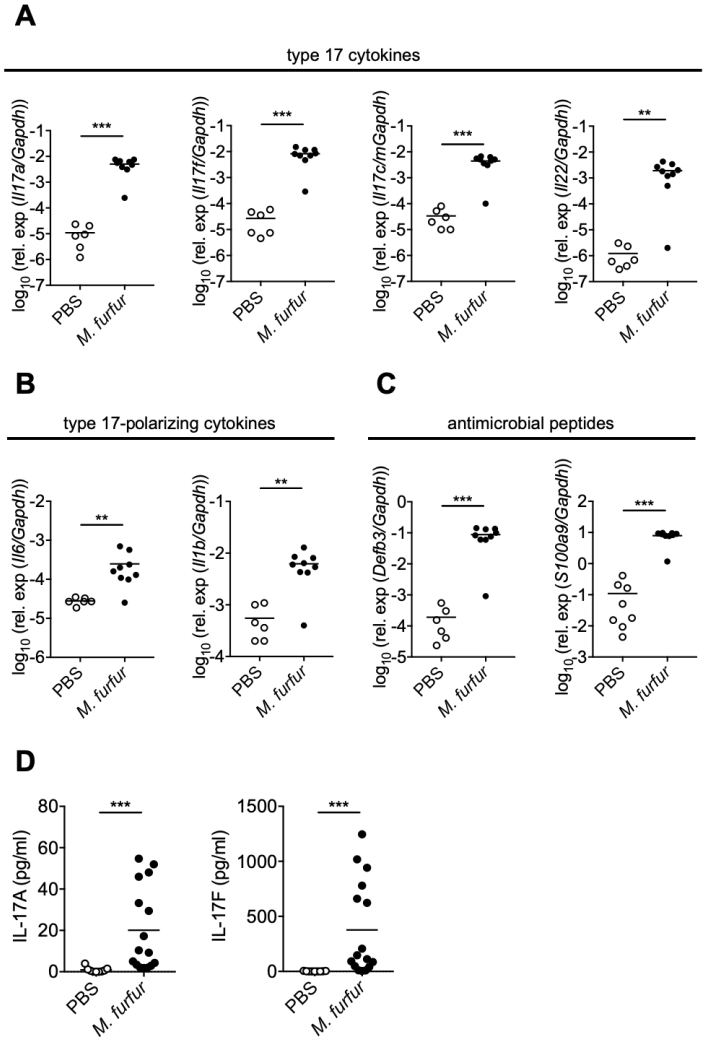

Figure 2. Epicutaneous exposure of *M. furfur* induces activation of the IL-17 pathway. A–C, mRNA expression levels of type 17 cytokines (Fig 2, *A*), type 17-polarizing cytokines (Fig 2, *B*) and antimicrobial peptides induced by IL-17 (Fig 2, *C*) in the lesional skin of C57BL/6J mice inoculated with *M. furfur* or treated with PBS for 4 days. IL-17A and IL-17F production (Fig 2, *D*) in the skin tissues of mice inoculated with *M. furfur* or treated with PBS for 4 days. Data represent combined results of two (Fig 2, *A–C*) and three (Fig 2, *D*) independent experiments. Each dot represents an individual mouse. **p<0.01, ***p<0.001 by Mann–Whitney U test.

Skin inflammation induced by *M. furfur* is IL-17-dependent

To evaluate the impact of IL-17 on *Malassezia*-induced skin inflammation, we infected mice doubly deficient in IL-17A and IL-17F (*Il17a*$^{-/-}$*f*$^{/-}$) as well as WT mice *with M. furfur* epicutaneously. On day 4 p.i., skin pathology assessed by skin disease score and epidermal thickness were significantly decreased in *Il17a*$^{-/-}$*f*$^{/-}$ mice compared with WT mice, without affecting the fungal load (Fig 3, *A–D)*. Reflecting the fungal loads, *Malassezia* yeasts were again localized in the cornified layers of the epidermis (Fig 3, *A*). These results indicated that IL-17A and IL-17F play an essential role in skin inflammation to *M. furfur* in the skin. *Rorc*$^{-/-}$ mice lacking IL-17-producing NKT cells,[28] Th17 cells,[16] and group 3 innate lymphoid cells (ILC3s)[15, 29, 30] showed little skin pathology following epicutaneous *M. furfur* infection and no epidermal thickening; similarly to *Il17a*$^{-/-}$*f*$^{/-}$ mice, there was no effect on fungal burdens (Fig 3, *A* and *E–G)*. Notably, production of IL-17A and IL-17F in lesional skin of *Rorc*$^{-/-}$ mice following fungal inoculation was significantly reduced compared with WT mice, and as expected, production of these cytokines in *Il17a*$^{-/-}$*f*$^{/-}$ mice was not detectable (Fig 3, *H* and *I*). These results suggested that the cellular sources of IL-17 in *Malassezia*-induced skin inflammation may be among the cell types absent in *Rorc*$^{-/-}$ mice.

ILC3s are involved in *M. furfur*-induced skin inflammation

To further confirm the cellular source of IL-17, we next used *Rag2*$^{-/-}$ mice, which lack T and B cells including Th17 cells and γδ T cells but possess ILC3s. *Rag2*$^{-/-}$ mice were depleted of ILC3s using an anti-CD90 mAb. Upon *Malassezia* infection, ILC3-depleted *Rag2*$^{-/-}$ mice showed significantly decreased skin disease scores and epidermal thickening compared with control mAb-treated *Rag2*$^{-/-}$ mice, again with no effect on

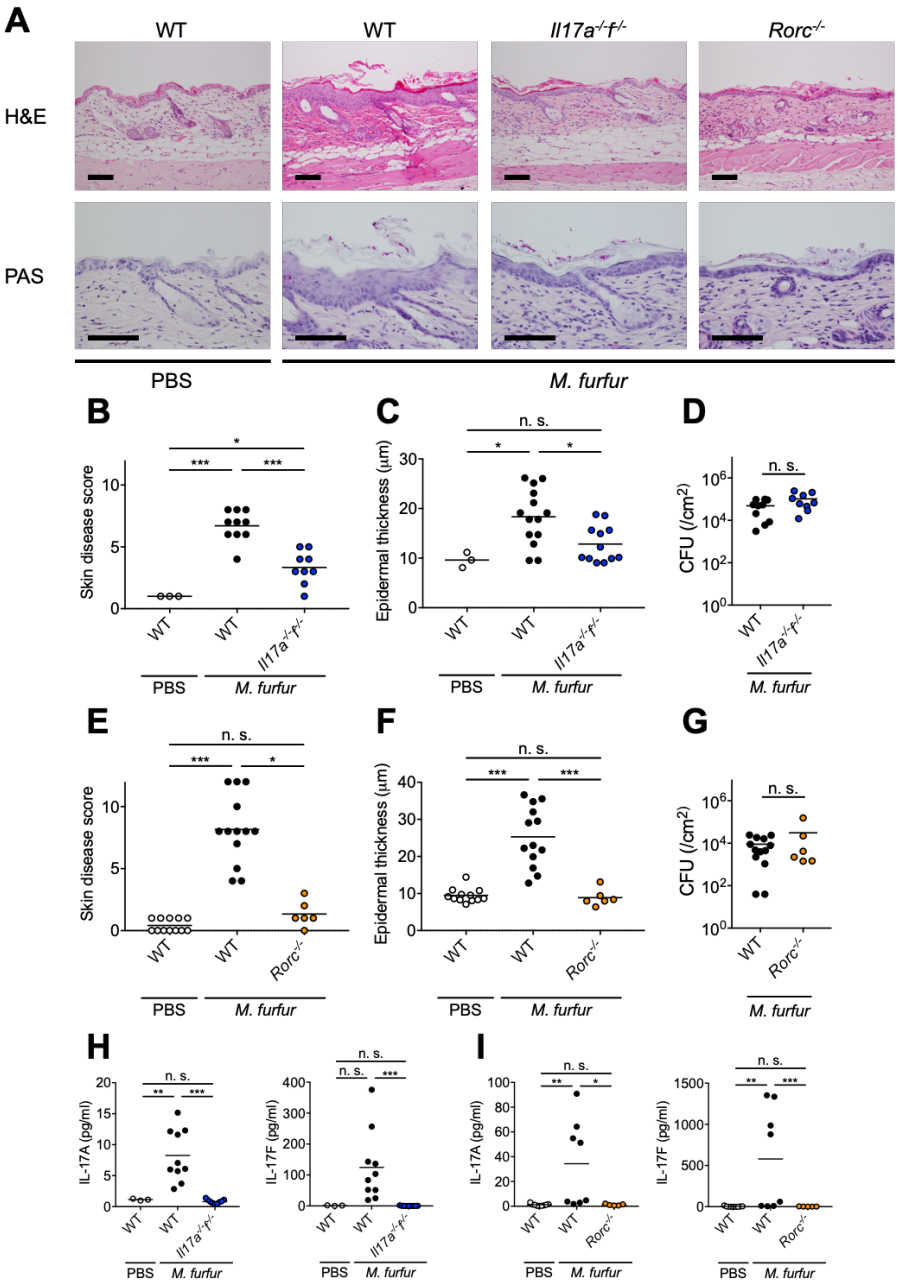

Figure 3. *M. furfur*-induced skin pathology is IL-17 dependent. A–I, C57BL/6J (WT), *Il17a*$^{-/-}$*f*$^{-/-}$ and *Rorc*$^{-/-}$ mice were epicutaneously inoculated with *M. furfur* for 4 days. WT mice were treated with PBS for 4 days for comparison. Representative H&E- and PAS-stained skin sections (Fig 3, *A*) are shown. Skin disease scores (Fig 3, *B* and *E*), epidermal thickness (Fig 3, *C* and *F*), *M. furfur* CFUs (Fig 3, *D* and *G*), and IL-17A and IL-17F production (Fig 3, *H* and *I*) in skin tissues are shown. Data represent combined results from two independent experiments (Fig 3, *B–I*). n.s., not significant; *p<0.05, **p<0.01, ***p<0.001 by Mann–Whitney U test (Fig 3, *D* and *G*), ANOVA with Tukey's multiple comparison test (Fig 1, *B* and *C*) or Kruskal–Wallis test with Dunn's multiple comparison test (Fig 1, *E, F, H,* and *I*).

fungal loads and localization of *Malassezia* yeasts in the cornified layers (Fig 4, *A–D*). Production of IL-17A and IL-17F in lesional skin of anti- CD90 mAb-treated *Rag2*$^{-/-}$ mice following fungal inoculation was significantly reduced compared with control mAb-treated *Rag2*$^{-/-}$ mice (Fig 4, *E*). Together, these results suggested that ILC3s were involved in *Malassezia*-induced skin inflammation as a source of IL-17.

Keratinocyte MyD88 is important for *M. furfur*-induced skin inflammation in an IL-17-dependent fashion

We next explored the upstream factors of *Malassezia*-induced skin inflammation. There is currently no information available regarding which receptors on epidermal cells directly interact with *Malassezia* yeasts except a few reports documenting that Toll-like receptor (TLR) 2 on human keratinocytes was implicated in sensing *Malassezia* yeasts and inducing an inflammatory response *in vitro*.[12, 31, 32] Therefore, we focused on MyD88, which is critical for signaling via members of the TLR/IL-1 receptor (TLR/IL-1R) superfamily. As shown in Fig 5 *A–C*, *Myd88*$^{-/-}$ mice showed significantly decreased skin disease scores and reduced epidermal thickening compared with WT mice upon *Malassezia* skin infection. Mice lacking MyD88 only in keratinocytes (*Myd88*$^{\Delta ker}$) also showed phenotypes similar to those of *Myd88*$^{-/-}$ mice with systemic deletion of MyD88. Fungal loads in both *Myd88*$^{\Delta ker}$ mice and *Myd88*$^{-/-}$ mice were indistinguishable from those of WT mice (Fig 5, *D*). Again, *Malassezia* yeasts were localized in the cornified layers (Fig 5, *A*). Remarkably, production of IL-17A and IL-17F was significantly reduced in *Myd88*$^{\Delta ker}$ mice and *Myd88*$^{-/-}$ mice compared with WT mice upon infection (Fig 5, *E*). These results indicated that MyD88 in keratinocytes was involved in *Malassezia*-induced IL-17-dependent skin

inflammation in this model.

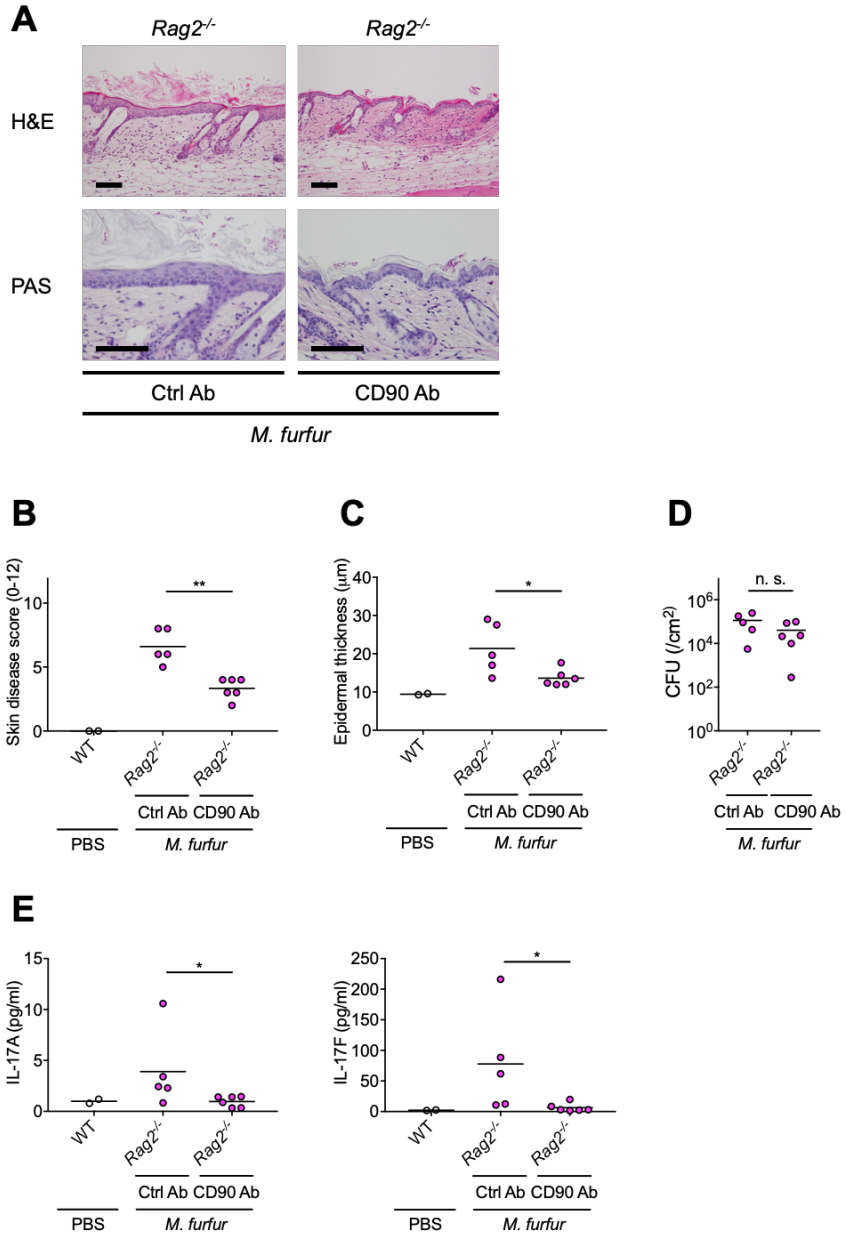

Figure 4. ILC3s contribute to *M. furfur*-induced skin inflammation. A–D, *Rag2*$^{-/-}$ mice treated with either anti-CD90 mAb or isotype-matched control mAb were epicutaneously inoculated with *M. furfur* for 4 days. Representative H&E- and PAS-stained skin sections (Fig 4, *A*) are shown. WT mice were treated with PBS for 4 days for comparison. Skin disease scores (Fig 4, *B*), epidermal thickness (Fig 4, *C*), *M. furfur* CFUs (Fig 4, *D*) and levels of IL-17A and IL-17F in the lesional skin (Fig 4, *E*) are shown. Data represent combined results from two independent experiments (Fig 4, *B*–*E*). Each dot represents an individual mouse. n.s., not significant; *p<0.05, **p<0.01 by Mann–Whitney U test.

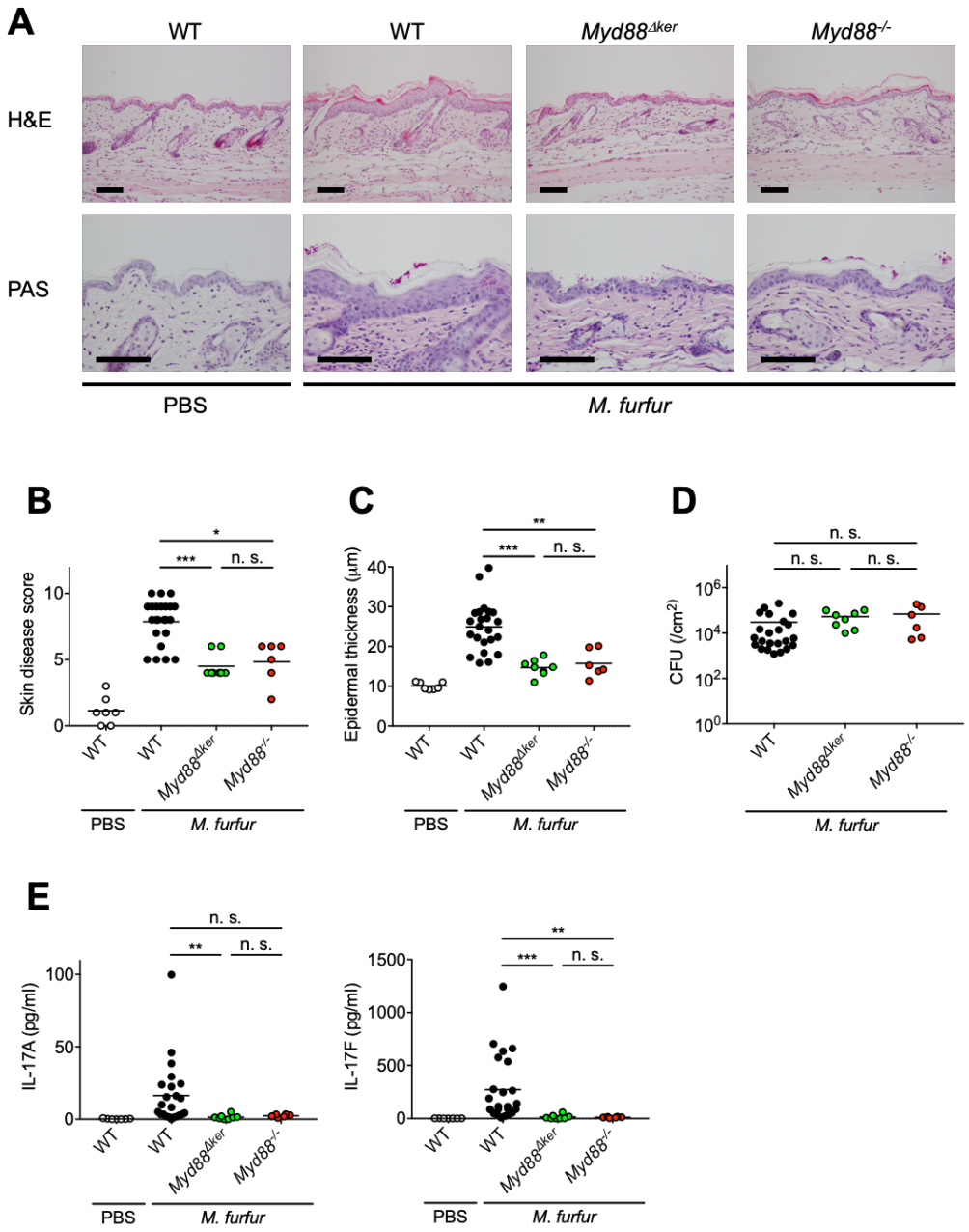

Figure 5. Keratinocyte MyD88 is involved in *M. furfur*-induced skin inflammation in an IL-17-dependent fashion. A–E, C57BL/6J (WT), *K14-CreMyD88$^{fl/fl}$* (*Myd88$^{\Delta ker}$*) and *Myd88$^{-/-}$* mice were epicutaneously inoculated with *M. furfur* for 4 days. WT mice were treated with PBS for 4 days for comparison. Representative H&E- and PAS-stained skin sections (Fig 5, *A*), skin disease scores (Fig 5, *B*), epidermal thickness (Fig 5, *C*), *M. furfur* CFUs (Fig 5, *D*) and levels of IL-17A and IL-17F in the lesional skin (Fig 5, *E*) are shown. Data represent combined results from four independent experiments. Each dot represents an individual mouse. n.s., not significant; *p<0.05, **p<0.01, ***p<0.001 by ANOVA with Tukey's multiple comparison test (Fig 5, *B*) or Kruskal–Wallis test with Dunn's multiple comparison test (Fig 5, *C to E*).

IL-36R, but not IL-1R, contributes to *Malassezia*-induced skin inflammation

MyD88 is a critical adaptor molecule in signaling mediated by multiple receptors belonging to the TLR/IL-1R superfamily.[33-36] In a preliminary experiment during the search for receptors that stimulated MyD88 in keratinocytes upon *Malassezia* infection, mice deficient in both TLR2 and TLR4 showed no significant reduction of *Malassezia*-induced skin inflammation compared with WT mice (data not shown). This result was inconsistent with previous reports documenting the involvement of TLR2 in human keratinocyte responses *in vitro*.[31, 32] Furthermore, mice deficient in IL-1R, which can be activated by either IL-1α or IL-1β, also showed no significant reduction in skin disease scores, epidermal thickening or fungal loads compared with WT mice (Fig 6, *A–C*). We next investigated the involvement of IL-36R, which also signals through MyD88,[37, 38] in *Malassezia*-induced skin inflammation by *in vivo* administration of a neutralizing mAb against IL-36R. WT mice receiving the neutralizing anti-IL-36R mAb showed significant reductions in skin disease scores and epidermal thickening compared with mice treated with a control mAb, with no effect on fungal loads (Fig 6 *D–F*). Production of IL-17A and IL-17F was significantly reduced in anti-IL-36R mAb-treated WT mice compared with mice treated with a control mAb (Fig 6, *G*). Together, these results indicated that IL-36R, but not IL-1R, contributed to *Malassezia*-induced skin inflammation. To investigate whether *Malassezia* induced expression of IL-36 transcripts, we incubated monolayers of primary differentiated keratinocytes with *M. furfur* and examined the expression of three agonistic cytokines of the IL-36 family (IL-36α, IL-36β, and IL-36γ). As shown in Fig 7, *A*, all transcripts were similarly upregulated when co-cultured with *Malassezia*, while viability of keratinocytes was comparable in the presence or absence of the fungi (Fig 7, *B*). These findings indicated

that keratinocytes have the potential to mediate IL-36 production upon the interaction with *Malassezia*.

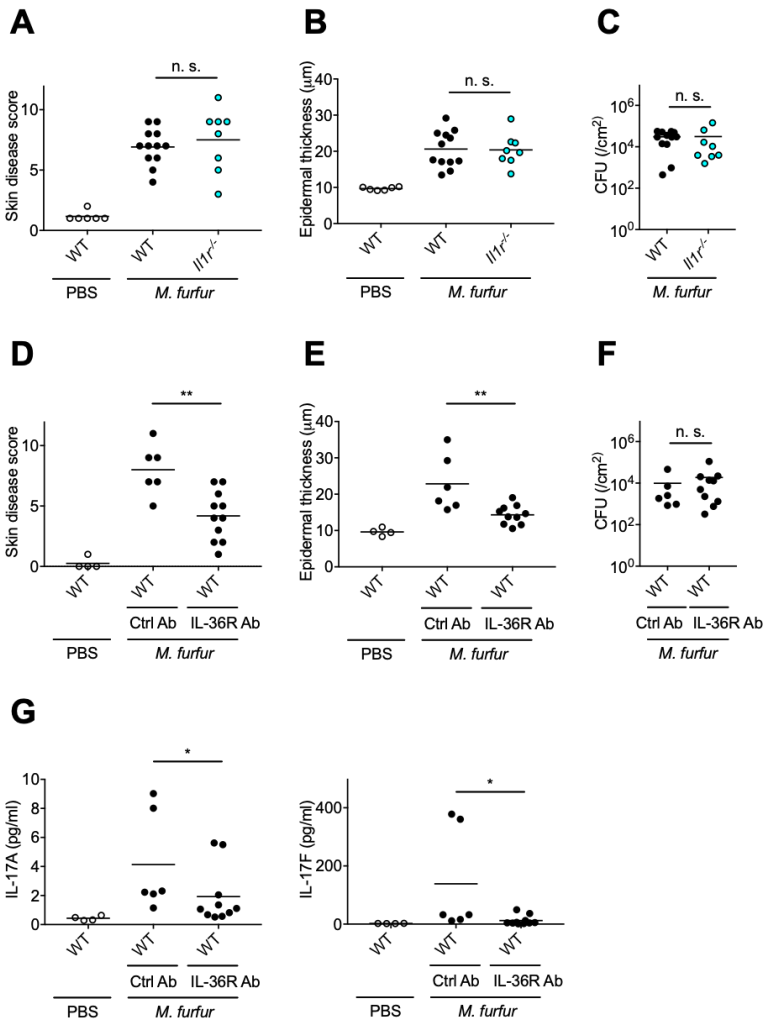

Figure 6. IL-36R, but not IL-1, contributes to *M. furfur*-induced skin inflammation. A–C, C57BL/6J (WT) and *Il1r*$^{-/-}$ mice were epicutaneously inoculated with *M. furfur* for 4 days. WT mice were treated with PBS for 4 days for comparison. Skin disease scores (Fig 6, *A*), epidermal thickness (Fig 6, *B*), and *M. furfur* CFUs in the skin (Fig 6, *C*) are shown. **D–F,** WT mice treated with either neutralizing anti-IL36R mAb or control mAb were epicutaneously inoculated with *M. furfur* for 4 days. WT mice were treated with PBS for 4 days for comparison. Mice from each group were evaluated for skin disease scores (Fig 6, *D*), epidermal thickness (Fig 6, *E*), *M. furfur* CFUs (Fig 6, *F*) and levels of IL-17A and IL-17F in lesional skin (Fig 6, *G*). Data represent combined results from two independent experiments (Fig, *A–G*). Each dot represents an individual mouse. n.s., not significant; *p<0.05, **p<0.01 by Mann–Whitney U test.

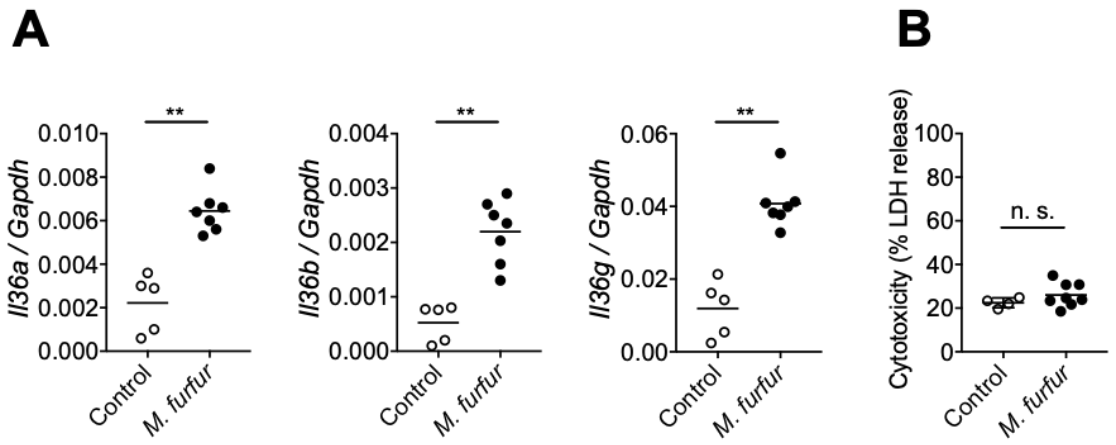

Figure 7. *M. furfur* induces IL-36 expression in keratinocytes. A and **B,** Primary differentiated keratinocytes from C57BL/6J mice were stimulated with live *M. furfur* for 24 h. The mRNA expression levels of IL-36 (IL-36α, IL-36β, and IL-36γ) (Fig 7, *A*) and cytotoxicity (Fig 7, *B*) of keratinocytes treated with or without *M. furfur* are shown. Data represent combined results from two independent experiments. n.s., not significant; **p<0.01 by Mann–Whitney U test.

DISCUSSION

Fungi of the genus *Malassezia* are lipophilic and require external lipids for growth. The genus currently comprises 18 *spp*., 12 of which have been identified on human skin.[39] *Malassezia spp.* are abundantly found on nearly all skin surfaces and are dominant commensal fungi of the human skin microbiota.[1, 2] *Malassezia spp*. are occasionally associated with many skin diseases ranging from diseases associated with mild inflammation (e.g., dandruff and pityriasis versicolor) to those associated with severe inflammation (e.g., seborrheic dermatitis, psoriasis and atopic dermatitis). Among *Malassezia spp.*, *M. sympodialis, M. globosa, M. furfur*, and *M. restrica* have frequently been isolated from skin lesions of patients, although their frequency of isolation varied from report to report.[40] However, there has been no clear evidence to document the roles of *Malassezia spp.* in the development of such skin diseases in human. To understand the roles played by *Malassezia* yeasts in their pathogenesis, it is necessary to dissect homeostatic immunity against *Malassezia spp.* under steady-state conditions and under inflammatory disease conditions. In other words, what is the initial impact of commensal *Malassezia* on the mammalian skin immune system under healthy conditions? To address this, we used the mouse, an animal that is not colonized by any *Malassezia spp.* We developed a new *Malassezia* infection model by inoculating the fungi onto the skin surface without barrier disruption and with no external lipid supplementation to investigate *Malassezia*-skin interactions and their role in the initial immune response against *Malassezia*. Working with this model, we revealed that *Malassezia*-induced skin inflammation, characterized by mild infiltration and epidermal thickening, was IL-17 dependent. We also found that ILC3s played a role in *Malassezia*-induced skin inflammation and that IL-17-dependent skin inflammation was mediated

by keratinocyte MyD88 signaling, in which the IL-36 receptor, but not the IL-1 receptor, was involved.

As a critical mediator of immune responses, IL-17 produced by a number of distinct immune cells such as Th17 cells, γδ T cells, NKT cells, NK cells, and ILC3s, is a vital cytokine in host defense against microorganisms including fungi (e.g., *Candida albicans*).[26, 41-43] In the skin, the importance of IL-17 for host defense against *C. albicans* cutaneous infection was shown by several inborn errors of immunity causing chronic mucocutaneous candidiasis in humans[44] and by several mouse models of *C. albicans* cutaneous infections.[45, 46] In addition *C. albicans*, *Microsporum canis* and *Trichophyton benhamiae*, the causative agents of superficial dermatophytosis, were also shown to induce IL-17-mediated immune responses (via Th1 and Th 17 cells) that were necessary to achieve fungal control.[26] These previous models required mechanical barrier disruption before inoculation of fungi and histopathologically showed prominent neutrophil infiltrates. More recently, it was shown that *Malassezia* triggered IL-17-mediated skin inflammation with massive infiltration of myeloid cells, including neutrophils, irrespective of skin barrier disruption before fungal inoculation. Moreover, dermal γδ T cells, but not dendritic epidermal T cells (DETCs), were primarily involved in IL-17-mediated skin inflammation in the early phase of inflammation, whereas memory Th17 cells contributed to the later acquired phase of inflammatory immunity upon the fungal infection.[6] In contrast, ILC3s were primary involved in IL-17-mediated skin inflammation in our *Malassezia* infection model. Although we did not examine the recall response at later time points, ILC3s under near-physiological conditions (e.g., no barrier disruption and no external lipid supplementation) may play a major role as the initial cells producing IL-17 during acute superficial *Malassezia*

skin infection. Innate lymphoid cells exist at barrier tissues (e.g., skin, intestine, and lung). In the skin, ILC3s are located in the dermis, where they contribute to the immunopathology of psoriasis as a significant source of IL-17 and IL-22.[47-50] Because *Malassezia* were reportedly abundant in psoriatic skin lesions, suggesting a potential role in triggering psoriasis plaque formation,[40, 51] it is intriguing to speculate that *Malassezia* may stimulate ILC3s to produce IL-17 and IL-22, contributing to the development of skin lesions in psoriasis. In this context, it is interesting to consider that our *Malassezia* infection model resembles psoriasis-like skin lesions without prominent neutrophil infiltration into the epidermis; similar lesions are frequently observed in the histopathology of psoriasis plaques. We may further consider that commensal *Malassezia* would trigger IL-17 production by ILC3s and provide a prerequisite condition for the development of the disease.

We found that mice lacking MyD88 only in keratinocytes (*Myd88*$^{\Delta ker}$) as well as *Myd88*$^{-/-}$ mice systemically deficient in MyD88 showed decreased skin inflammation upon superficial *Malassezia* skin infection, but comparable fungal loads, compared with WT mice. Importantly, in both types of MyD88-deficient mice, IL-17 production was dramatically reduced upon infection. Together, these results indicated that keratinocyte MyD88 signaling played a major signaling role in the direct interaction between *Malassezia* and keratinocytes. These results were inconsistent with the observations reported by Sparber *et al*. documenting that the IL-17A transcripts in the lesional skin of *Myd88*$^{-/-}$ mice on day 2 p.i. by *Malassezia* with barrier disruption were indistinguishable from those of WT mice.[6] The discrepancy may reflect differences in the *Malassezia* species used, the infection site, the timing of observation, or the presence or absence of initial skin barrier disruption.

Regarding the upstream receptors involved in MyD88 signaling, we found that IL-36R, but not IL-1R, was involved in superficial *Malassezia* skin infection in our model. By contrast, IL-1R and IL-36R signaling were both involved in murine acute oral candidiasis.[52, 53] In addition, we recently found that IL-1R and IL-36R signaling were both critical for *Staphylococcus aureus*-induced skin inflammation.[23] One potential explanation of these differences is that these pathogens might induce expression of distinct proteases derived by host immune cells (e.g., neutrophils and mast cells) to differentially activate members of the IL-1/IL-36 family.[54] We speculate that cells other than neutrophils may be cellular sources of these proteases because the neutrophil infiltrates observed during *Malassezia*-induced skin inflammation were much milder than those observed in *S. aureus*-induced skin inflammation or oral candidiasis. However, further study is needed to clarify why IL-1R is apparently not involved in *Malassezia*-induced skin inflammation in our model.

In summary, the present study revealed that *Malassezia* induce IL-17-dependent skin inflammation that is mediated by IL-36R/MyD88 signaling in keratinocytes. It is important to note several unsolved key questions. How does *Malassezia* induce IL-36R-mediated responses through keratinocyte MyD88 signaling? Is its production induced by soluble factor(s) derived from *Malassezia* or their cell wall component(s)? How does host immunity maintain homeostasis against this commensal fungus at the skin surface? How are *Malassezia* involved in the development of *Malassezia*-associated skin diseases given continuous existence on the skin? Further studies are required to address these questions. Nevertheless, we believe that the present study provides insight into the interactions between the host and commensal *Malassezia* yeasts on the skin, and also provide clues toward understanding their contributions to the pathogenesis of

Malassezia-associated skin diseases including psoriasis and atopic dermatitis. Notably, the IL-36R/ MyD88 signaling pathway may become a target to identify virulence factors of *Malassezia*, eventually leading to the development of therapeutic interventions for those disorders.

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