IL-17 produced by ILC3 and γδ T cells is pivotal for host defense against skin candidiasis *via* neutrophil activation

（ILC3とγδT cellsから産生されるIL-17は、皮膚カンジダ症感染防御において、好中球活性化を介して極めて重要な役割を演じている）

千葉大学大学院医学薬学府

先端生命科学専攻

（主任：松江弘之教授）

岩澤　真理

SUMMARY

IL-17 is important in systemic and mucosal *Candida albicans* infections, but its role in skin candidiasis remains poorly understood. Using a model of epicutaneous candidiasis without skin abrasion, we found impaired fungal clearance and marked skin inflammation in mice lacking both interleukin-17A and F. Group 3 innate lymphoid cells (ILC3) and γδ T cell were the major skin IL-17 producers in response to *C. albicans*. IL-17A and F promoted neutrophil recruitment into the epidermis and the expression of antimicrobial peptides. Notably, phagocytosis of hyphal forms of *C. albicans* was severely impaired in IL-17-deficient neutrophils but restored by stimulation with recombinant IL-17. Fungal recognition receptors TLR2, Dectin-1, Dectin-2, and the adaptor signaling molecule MyD88, FcRγ, and Card9 were dispensable for clearance of *C. albicans*. These findings indicate that IL-17 from ILC3 and γδ T cells plays a pivotal role in cutaneous host defense by recruiting and activating neutrophils to phagocytose and eradicate *C. albicans* in the epidermis.

INTRODUCTION

Candida albicans, a commensal fungus that is part of the human microbiota in the skin and the intestine, causes a wide spectrum of infections from mild to severe and often lethal disease in the setting of immunosuppression and inherited immunodeficiency. For example, patients with genetic defects in the fungal recognition C-type lectin receptor Dectin-1 or its downstream signaling components, T helper 17 (Th17) cell differentiation, and IL-17 signaling develop chronic mucocutaneous candidiasis (CMC) (Ling et al., 2015; Milner and Holland, 2013). In contrast, HIV-infected patients with severe CD4 T-cell deficiency commonly develop oropharyngeal and esophageal candidiasis (OPC) rather than CMC (Cassone and Cauda, 2012) and systemic candidiasis predominantly occurs in neutropenic patients (Martino et al., 1989). IL-23 and IL-17-mediated signaling and neutrophil activation are important for host defense against cutaneous *C. albicans* infection in intradermal infection models (Conti et al., 2015; Kagami et al., 2010; Urban et al., 2009). In OPC models, IL-17 derived from Th17 cells, γδ T cells and ILC3 is important for protective immunity against *C. albicans* (Conti et al., 2014; Gladiator et al., 2013). In the mouse skin, IL-17 is produced by several cell types including dendritic epidermal T cells (DETCs), dermal γδ T cells, $CD4^+$ and $CD8^+$ T cells and ILC3 (Gray et al., 2011; MacLeod et al., 2013; Naik et al., 2015). Furthermore, $CD4^+$T cells or γδ T cells are major IL-17-producers in epicutaneous candidiasis (ECC) models in which *C. albicans* infection is induced after mechanical disruption of the epidermis (Hirota et al., 2011; Kashem et al., 2015a; Kashem et al., 2015b). However, the role of IL-17 and the IL-17-producing cells is unclear in more natural infection conditions without mechanical disruption of the epidermis.

IL-17 orchestrates neutrophil migration and activation. For example, IL-17 induces chemokines such as CXC-chemokine ligand 8 (also known as IL-8) and cytokines including interferon-γ, tumor necrosis factor (TNF)-α and granulocyte-macrophage colony stimulating factor (GM-CSF) that activate neutrophils (Mantovani et al., 2011). Neutrophils play a critical role in the clearance *C. albicans* from infected sites *via* the production of reactive oxygen species. Furthermore, S100A8/S100A9 proteins complex (also known as calprotectin) released in neutrophil extracellular traps (NETs) played an important role in host defense against *C. albicans* in systemic and subcutaneous infection (Urban et al., 2009). In previous ECC models, the skin was disrupted by scratching with needles or rubbing with sandpaper prior to *C. albicans* inoculation to allow the *C. albicans* to be directly recognized by dermal cells (Hirota et al., 2011; Kashem et al., 2015a; Kashem et al., 2015b). However, in cutaneous candidiasis, *C. albicans* typically resides in the cornified layer of epidermis, and neutrophil-rich microabscess formation is mainly observed in the subcorneal layer of the epidermis after infection (Elder et al., 2005). In order to study *C. albicans* infection that more closely recapitulates physiological skin infections, we developed an ECC mouse model that did not require any epidermal disruption. In this report, we found that IL-17 plays a critical role in the clearance of *C. albicans* from the infected skin. In contrast to the previous reports, we found that γδ T cells and ILC3 are the major sources of IL-17 in response to ECC. In addition, our data indicate that IL-17 secreted from ILC3 and γδ T cells can directly control neutrophil activation, resulting in the enhanced migration and phagocytosis by neutrophils in the infected skin.

RESULTS

IL-17 signaling is essential for the clearance of *C. albicans* in ECC. To understand the role of IL-17 in controlling *C. albicans* under physiological conditions, we developed a novel ECC mouse model, in which the dorsal skin of mice was colonized with *C. albicans* without disruption of the epidermis (**Figure 1A**). In wild-type mice, the skin inflammation was subsided (**Figure 1B**) and most *C. albicans* were cleared on day 7 after colonization, as measured by the number of colony-forming units (CFU) in the skin tissue (**Figure 1C**). To assess the role of IL-17A and IL-17F in our ECC model, we infected mice deficient in IL-17A (*Il17a*$^{-/-}$), IL-17F (*Il17f*$^{-/-}$), doubly deficient mice (*Il17af*$^{-/-}$) or wild-type mice. On day 2 after *C. albicans* colonization, histological analysis showed parakeratosis and marked neutrophil-rich inflammatory infiltrates with evidence of hyphal forms of *C. albicans* in the cornified layer and the fungal burden was comparable in *Il17a*$^{-/-}$, *Il17f*$^{-/-}$, *Il17af*$^{-/-}$ and wild-type mice (**Figures 1C and S1**). In contrast, only *Il17af*$^{-/-}$ mice developed severe neutrophil-rich skin inflammation which was associated with marked impairment in fungal clearance, with ~ 3 logs higher *C. albicans* loads than in wild-type, *Il17a*$^{-/-}$ or *Il17f*$^{-/-}$ mice on day 7 (**Figures 1B and 1C**). To assess the expression of IL-17A and F in the ECC model, we measured cytokine mRNA and protein expression levels in the lesional skin on day 2 after *C. albicans* colonization. Both *Il17a* and *Il17f* mRNA as well as the proteins were induced in the lesional skin of wild-type mice after infection, and the increased mRNA and proteins of IL-17F and IL-17A were also detected in the lesional skin of *Il17a*$^{-/-}$ and *Il17f*$^{-/-}$ mice complementary (**Figure 1D and 1E**). Notably, several proinflammatory cytokines and chemokines including IL-1β, IL-6, TNF-α, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP) -1α and MIP-1β were induced in the

lesional skin of wild-type mice on day 2 after *C. albicans* colonization which was largely comparable to that observed in *Il17af*$^{-/-}$ mice (**Figure S2**). Wild-type and *Il17af*$^{-/-}$ mice showed similar skin commensal composition (**Figure S3**), suggesting that phenotypic difference between wild-type and *Il17af*$^{-/-}$ mice in the ECC model was not mediated through the regulation of commensal bacteria. Taken together, these results indicate that both IL-17A and IL-17F played an essential and redundant role in host defense against *C. albicans* in the ECC model.

ILC3 and γδ T cells are the primary source of IL-17 in ECC. To identify the cellular source of IL-17A in our ECC model, we analyzed immune cell populations in the lesional skin using IL-17A-eGFP reporter mice. On day 2 after *C. albicans* colonization, IL-17A-eGFP^{+} cells in the CD45^{+} cell population were increased after *C. albicans* infection when compared to control mice (**Figure 2A**). The IL-17A-eGFP^{+} cells were only found in the CD90^{+} cell population which consisted of 22.3% of Lineage^{-} (CD3e, CD11b, B220, Ly-76, Ly-6G and Ly-6C) cells and 74.1% Lineage^{+} cells (**Figure 2A**). Notably, 72.4% of the IL-17A-eGFP^{+} cells were TCRγδ^{+} cells and 23.4% were TCRγδ^{-} TCRβ^{-} cells (**Figure 2A**). Although a previous study showed that neutrophils can produce IL-17A in *Aspergillus* infection models (Taylor et al., 2014), IL-17A-eGFP^{+} cells were not found in neutrophils (Gr-1^{+}) in our ECC model (**Figure 2B**). Furthermore, IL-17F^{+} CD90^{+} Lineage^{-} ILC3 and IL-17F^{+} γδ T cells, but not Gr-1^{+} neutrophils, were detected by intracellular IL-17 staining after epicutaneous *C. albicans* infection（**Figures 2C, 2D and S4**）. Analysis of Vγ1.1, Vγ2 and Vγ3 TCR expression in the CD45^{+}CD90^{+} population revealed a comparable pattern of expression in IL-17A- and IL-17F-positive cells on day 2 after *C. albicans* colonization, suggesting that Vγ3^{+}

DETCs, Vγ2$^+$ dermal $\gamma\delta$ T cells and peripheral Vγ1.1$^+$ $\gamma\delta$ T cells are all induced to produce IL-17A and F in the ECC model (**Figures S4A and S4B**). Taken together, these results indicate that IL-17A and IL-17F are produced by ILC3 and several $\gamma\delta$ T cells including DETCs, but not neutrophils, in response to epicutaneous *C. albicans* infection.

ILC3 cells are essential for host defense against ECC. We next addressed the role of ILC3, which are able to produce IL-17A and IL-17F, in our ECC model. To test this, we compared *Rag2*$^{-/-}$ mice that lack T and B cells including $\gamma\delta$ T cells, but have ILC3 cells and *Rag2Il2rg*$^{-/-}$ mice that lack both $\gamma\delta$ T cells and ILC3 cells. Similar to *Il17af*$^{-/-}$ mice, *Rag2Il2rg*$^{-/-}$ mice but not *Rag2*$^{-/-}$ mice developed severe skin inflammation associated with high fungal burdens on day 7 after *C. albicans* colonization (**Figures 3A and 3B**). Importantly, small amounts of IL-17A were detected in *Rag2*$^{-/-}$ mice on day 2 after infection, while production of both IL-17A and IL-17F was abolished in *Rag2Il2rg*$^{-/-}$ mice (**Figures 3C and 3D**). Furthermore, flow cytometry analysis revealed that *Rag2Il2rg*$^{-/-}$ developed neither IL-17A$^+$ nor IL-17F$^+$ cells in the lesional skin on day 2 after infection (**Figure S5**). These results suggest that IL-17-producing ILCs are essential in host defense against *C. albicans* of our ECC model**.**

Dectin-1, Dectin-2, Card9, TLR2, and FcRγ are dispensable for recognition of *C. albicans* in ECC. The cell wall of fungi including *C. albicans* is mainly composed of multiple layers of carbohydrates, including mannans, β-glucans, proteoglycans and chitins (Saijo and Iwakura, 2011). The C-type lectin receptors Dectin-1 and Dectin-2 are specific receptors for β-glucans and α-mannan, respectively, and mediate the induction of inflammatory cytokines through activation of the caspase-recruitment domain family,

member 9 (Card9) (Hara et al., 2007; Saijo et al., 2007; Saijo et al., 2010). Proteoglycans in fungal cell walls are recognized by Toll-like receptor 2 (TLR2) and TLR6 heterodimers (Underhill et al., 1999). To identify which receptor is responsible for the recognition of *C. albicans* in our ECC model, we analyzed *Tlr2*$^{-/-}$, *Myd88*$^{-/-}$, *Clec7a*$^{-/-}$*Clec4n*$^{-/-}$ (Dectin-1 and Dectin-2 deficient), *Fcer1g*$^{-/-}$ and *Card9*$^{-/-}$ mice. In contrast to systemic fungal infection models (Hara et al., 2007; Saijo et al., 2007; Saijo et al., 2010; Yanez et al., 2010), mice deficient in TLR2, Myd88, Dection1/2, Fc receptor γ (FcRγ) which lack the Fc receptor chain γ required for assembly and signal transduction for Dectin-2, Mincle and MCL, or Card9 did not display any defects in the clearance of *C. albicans* in the ECC model compared to wild-type mice (**Figures 4A and 4B**). These results suggest that clearance of *C. albicans* by cells in the epidermis is either independent of these known receptors involved in fungal recognition, or there is remarkable redundancy among these receptors in the recognition and clearance of *C. albicans*.

IL-17 signaling recruits neutrophils and induces phagocytosis in the *C. albicans* ECC model. Neutrophils are critical in controlling fungal infection including candidiasis that often causes lethal infections in neutropenic patients (Arendrup, 2010). However, the role of IL-17 in neutrophil activation in *C. albicans* infection remains controversial. The S100A8/S100A9 complex released in NETs is important for host defense against *C. albicans* in systemic and subcutaneous infection, but a link to IL-17 has not been investigated (Urban et al., 2009). We found that both *S100a8* and *S100a9* mRNA levels in lesional skin were dramatically decreased in *Il17af*$^{-/-}$ mice compared to that in wild-type, *Il17a*$^{-/-}$ and *Il17f*$^{-/-}$ mice (**Figure 5A**). Immunofluorescence staining

demonstrated that S100A9 was expressed in Ly6G^{+} and myeloperoxidase (MPO)$^{+}$ neutrophils located in the lesional skin of wild-type mice on day 2 after inoculation (**Figure 5B**). In contrast, reduced numbers of Ly6G^{+} MPO^{+} S100A9^{+} neutrophils were found in the lesional skin of *Il17af*$^{-/-}$ mice (**Figure 5B**). Radial hyphae growth of *C. albicans* observed at 37°C culture was dramatically reduced after co-culture with wild-type mice neutrophils, but such a reduced growth was not observed in co-culture with *Il17af*$^{-/-}$ mice neutrophils (**Figures 5C and D**). In contrast, yeast form of *C. albicans* obtained at 30°C culture was not recognized by wild-type mice neutrophils (**Figure S6A**). Importantly, the loss of ability of *Il17af*$^{-/-}$ neutrophils to inhibit hyphae growth was restored by IL-17A stimulation (**Figures 5C and D**). Notably, neutrophils from *Il17af*$^{-/-}$ mice exhibited impaired migration and binding to *C. albicans* compared wild-type neutrophils (**Supplementary Movie 1**). Furthermore, neutrophils from *Il17af*$^{-/-}$ mice expressed similar amounts of *Clec7a, S100a9* mRNA and S100A9 protein compared to wild-type neutrophils, indicating that IL-17 does not affect the expression of Dectin-1 and S100A9 in neutrophils (**Figure S6B**). In addition, NETs release by neutrophils in response to hyphal forms was comparable in wild-type and *Il17af*$^{-/-}$ neutrophils (**Figure S6C**). Reactive oxygen species (ROS) secretion was also comparable in wild-type and *Il17af*$^{-/-}$ neutrophils (**Figure S6D**). To confirm the importance of neutrophils in the ECC model, we depleted neutrophils in wild-type mice by administration of anti-Ly6G antibody and colonized the skin of treated mice with *C. albicans* (**Figure 6A**). Ly6G antibody-treated mice, but not isotype IgG-treated mice, exhibited high fungal burdens on day 7 after inoculation which was comparable to that observed in *Il17af*$^{-/-}$ mice (**Figure 6C**). Of note, Ly6G antibody-treated mice had milder skin inflammation than infected *Il17af*$^{-/-}$ mice on day 7 after colonization presumably

due to systemic depletion of neutrophils (**Figure 6B**). These results indicate IL-17A and IL-17F plays a pivotal role in host defense against ECC by recruiting neutrophils and regulating phagocytosis activity in neutrophils.

DISCUSSION

There is ample evidence that IL-17, produced by several cell types including Th17, γδ T cells, $CD8^+$ T cells and ILC3, is important for host defense against microorganisms including *C. albicans* in the skin and other organs (Bar et al., 2014; Cho et al., 2010; Conti and Gaffen, 2015; Conti et al., 2014; Kagami et al., 2010; Kashem et al., 2015b; McDonald, 2012). However, the host immune mechanisms that regulate *C. albicans* invasion in the epidermis remain poorly understood because previous models of ECC involved mechanical disruption of the epidermis prior to infection or direct inoculation of fungi in the dermis. We show here that IL-17A and IL-17F produced by ILC3 and γδ T cells, including $V\gamma3^+$ DETCs, $V\gamma2^+$ dermal γδ T cells, peripheral $V\gamma1.1^+$ γδ T cells, are important for preventing the growth of *C. albicans* in our ECC model in which hyphal forms of *C. albicans* are mainly found in the cornified layer of epidermis. *Il17af*$^{-/-}$ mice, but not *Il17a*$^{-/-}$ and *Il17f*$^{-/-}$ mice, developed severe skin inflammation with high fungal burden on day 7 after *C. albicans* inoculation, suggesting that IL-17A and IL-17F act redundantly to regulate the eradication of *C. albicans*. Our results are in contrast with, a previous study using an ECC model involving mechanical disruption of the epidermis, that showed increased burdens of *C. albicans* in *Rag1*$^{-/-}$ and *Tcrd*$^{-/-}$ mice and γδ T cells as the major IL-17A-producing cell type in host defense against *C. albicans* in the skin (Kashem et al., 2015b). In that model, however, *C. albicans* was recognized by $TRPV1^+$ nociceptive receptors, which led to production of IL-23 and IL-17A (Kashem et al., 2015b). The $TRPV1^+$ nociceptive receptors act as pain and temperature recognition receptors (Abrahamsen et al., 2008) that are important in psoriasic skin inflammation in mice (Riol-Blanco et al., 2014). However, $TRPV1^+$ nociceptive receptors can be identified in the dermis (Riol-Blanco et al., 2014), although the cell

component of *C. albicans* recognized by TRPV1^{+} receptor remains unknown. How *C. albicans* is recognized by host cells in ECC and especially in the cornified layer of epidermis remains unclear. To address this question, we tested mice deficient in known fungal sensors, including TLR2, MyD88, Dectin-1, Dectin-2, FcRγ and the signaling molecule Card9 in our ECC model. In contrast to systemic, mucosal and cutaneous models with epidermal disruption, the eradication of *C. albicans* was unperturbed in all mice deficient in the fungal sensors tested, indicating that unknown receptors and/or redundancy among receptors are important in the recognition of *C. albicans* in the epidermis. Further analysis is needed to understand how *C. albicans* is recognized in the superficial layer of epidermis.

Neutrophils migrate into the epidermis and form neutrophil-rich microabscesses in the subcorneal layer of the epidermis in human skin infected with *C. albicans* which is also observed in our mouse ECC model. We found that *S100A8* and *S100A9* expression was greatly reduced in lesional skin of *Il17af*$^{-/-}$ mice, but not *Il17f*$^{-/-}$ and *Il17a*$^{-/-}$ mice after fungal colonization. The S100A8/S100A9 complex is known to be expressed in epithelial cells, monocytes and granulocytes (Hobbs et al., 2003). Notably, we found that massive infiltration of S100A9 expressing Ly-6G^{+}MPO^{+} neutrophils within the infected epidermis was observed in wild-type mice but not in *Il17af*$^{-/-}$ mice on day 2.On day 7, *Il17af*$^{-/-}$ mice showed significant neutrophil migration in the infected epidermis but still colonized with high fungal burden while wild-type mice had already eliminated *C. albicans* from the skin, suggesting that IL-17 signaling might be important for both recruitment and activation of neutrophils in ECC. Cytokines and chemokines regulated by IL-17, such as G-CSF, CXC chemokines (CXCL1, CXCL2, CXCL5, CXCL8), TNF-

α, IFN-γ and S100A8/S100A9 complex are known to orchestrate the recruitment and activation of neutrophils (Pelletier et al., 2010; Shen et al., 2006). A key characteristic of *C. albicans* is its ability to change from yeast to hyphal morphology in ECC. We found that isolated neutrophils from *Il17af*$^{-/-}$ mice exhibited a marked impairment in inhibiting hyphal growth of *C. albicans* which was restored by addition of recombinant IL-17. The reduced ability of *Il17af*$^{-/-}$ neutrophils to ingest hyphal forms of *C. albicans* was not explained by reduced Dectin-1 and S100A9 expression, NET release and ROS production. In contrast, *in vitro* live imaging experiment revealed that *Il17af*$^{-/-}$ neutrophils were not able to recognize hyphal growth, attach and phagocyte *C. albicans* while wild-type neutrophils quickly attached and phagocyte the hyphal forms of *C. albicans.* These results indicate that IL-17A and F are important for migration, sensing and phagocytosis of hyphal forms in neutrophils. Moreover, in these *in vitro* experiments, wild-type neutrophils could be only be exposed to low steady state amounts of IL-17 which is sufficient for activation compared with *Il17af*$^{-/-}$ neutrophils. These results provide a plausible explanation to account for the observation that *C. albicans* is clear from the epidermis in *Il17a*$^{-/-}$, *Il17f*$^{-/-}$ and *Rag2*$^{-/-}$ mice that can produce small amounts of IL-17 cytokines in the infected skin. Depletion of neutrophils resulted in marked impairment of *C. albicans* which was comparable to that observed in *Il17af*$^{-/-}$ mice in our ECC model.

In summary, we have developed a novel ECC mouse model that better recapitulates physiological candidiasis in the skin. Using this model, we show that IL-17A and F produced by both ILC3 and γδ T cells play a critical role to clear *C. albicans* from the epidermis. Importantly, in contrast to previous skin-damaged ECC models, different

types of cells were identified as IL-17A and F-producers in our model. In addition, we found that recruitment and activation of neutrophils through IL-17 signaling was essential for host defense against *C. albicans* in the epidermis in our ECC model. Because none of the known receptors or adaptor molecules involved in fungal recognition was found essential our ECC model, it will be important to identify new mechanism associated with IL-17 that regulate the eradication of *C. albicans* by host cells in the cornified or upper layer of the epidermis.

EXPERIMENTAL PROCEDURES

Mice

Wild-type mice on C57BL/6J background were purchased from Japan CLEA Inc (Tokyo, Japan). *Il17a*$^{-/-}$, *l17f*$^{-/-}$, *Il17af*$^{-/-}$, *Card9*$^{-/-}$, and IL-17A-eGFP reporter mice were previously reported (Saijo et al., 2010). *Clec7aClec4n*$^{-/-}$ were generated by crossing *Clec7a*$^{-/-}$ mice with *Clec4n*$^{-/-}$ mice, *Tlr2*$^{-/-}$, *Myd88*$^{-/-}$ were purchased from Oriental Yeast Co., LTD. (Tokyo, Japan). *Rag2Il2rg*$^{-/-}$ mice were provided by Dr. S. Koyasu (Laboratory for Immune Cell Systems, RIKEN Research Center for Integrative Medical Sciences) or purchased from Taconic (New York, NY). *Fcer1g*$^{-/-}$ mice were purchased from Taconic (New York, NY). *Rag2*$^{-/-}$ mice were kindly provided by Dr. S. Sakaguchi (Osaka University, Osaka Japan) or purchased from Taconic. All mice were on C57BL/6 background. 6-12 week-old female mice were used for experiments and age and gender-matched wild-type C57BL/6J mice were used as controls. All mice were kept under specific pathogen-free conditions at Chiba University. All experiments were performed in strict accordance with the regulation of Animal Experimentation at Chiba University. The protocol was approved by the Chiba University Institutional Animal Care and Use Committee.

Culture of *C. albicans*

C. albicans (SC5314) was cultured for 48-96 hours at 30°C on potato dextrose agar (PDA) plates (Difco). Single colonies were picked up into sterile PBS and re-suspended at 10^8 CFU/ml in PBS immediately before inoculation.

C. albicans infection

The dorsal skin of mice was shaved with an electric clipper, applied hair removing

cream (Veet® In Shower Hair Removal Cream) 4 days prior to the infection. One hundred microlitres of the yeast form of *C. albicans* strain SC5314 suspension (10^7 CFU) was placed on a patch of sterile gauze (1 x 1 cm) and attached to the shaved skin with transparent bioocclusive dressing (Tegaderm; 3M). Each mouse was exposed to *C. albicans* for 2 or 7 days through the patch. Mice were checked every day and re-wrapped with Tegaderm film when necessary. Mice were sacrificed and the skin samples were used for skin histology, CFU assay, RNA extraction, cytokine titration, and flow cytometric analysis.

Skin Histology and Immunofluorescence Staining

Skin tissue was formalin fixed, paraffin embedded, and sectioned for hematoxylin and eosin (HE) and Periodic acid-Schiff stain (PAS). For immunofluorescence staining, paraffin embedded skin samples were deparaffinized, antigen reactivated and then incubated with primary antibodies.

Mouse Skin CFU and cytokine titrations

The infected area (5 mm^2) was homogenized in sterile PBS and serially diluted onto PDA plates with 100 mg/L of chloramphenicol (Sigma-Aldrich) and incubated at 30°C for 48-72 hrs to determine CFU. Then, the homogenates were subjected to the determination of cytokines with a Cytometric Bead Array System (BD Biosciences).

Analysis of Skin Microbiota Composition

Wild-type and *Il17af*$^{-/-}$ mice were co-housed for 5 weeks and then separated into different cages. Five weeks after separation, feces and skin samples were collected and bacterial DNA were extracted by E.Z.N.A. Stool DNA Kit (OMEGA bio-tek). The V4

legion of the 16S rRNA gene (252 bp) was sequenced with Illumina Miseq sequencer, and =22,000 reads were analyzed by Mothur. OTUs were classified into taxonomic phylotype at >97% identity and analyzed by Mothur.

RNA Extraction from Skin Samples and q-PCR

Total skin was homogenized and mRNA was extracted by EZNA® Total RNA Kit I. Total RNA was used to synthesize cDNA using High Capacity RNA- to-cDNA Kit (Applied Biosystems). Relative quantification of indicated genes was determined by real-time PCR using SYBR Premix Ex Taq II (Takara Bio Inc.) normalized to the expression of *Gapdh*. Quantitative reverse transcription PCR was performed using the Applied Biosystems® StepOnePlus™ Real-Time PCR Systems. The primer sequences were as follows: *Gapdh*, 5'-TGTGTCCGTCGTGGATCTGA-3' and 5'-TTGCTGTTGAAGTCGCAGGAG-3'; *Il17a*, 5'-CTGTGTCTCTGATGCTGTTG-3' and 5'-ATGTGGTGGTCCAGCTTTC-3'; *Il17f*, 5'-CTGTTGATGTTGGGACTTG-3' and 5'-GTTCATGGTGCTGTCTTCCTG-3'.

Preparation of Skin Immune Cells and Flow Cytometric Analysis

The total skin was collected on day 2 after *C. albicans* inoculation and single cell suspension was prepared as previously described (Kabashima et al., 2003). Briefly, single cell suspensions from skin were prepared 2 day after infection and stained with the indicated antibodies (Abs). For the preparation of single-cell suspensions from skin, the infected skin was processed with 2 U/ml of Dispase II (Sigma-Aldrich) in RPMI 1640 medium (Signa-Aldrich) for 1 h at 37°C, and then incubated for 1 h at 37°C with RPMI containing 2 mg/ml Collagenase II (Sigma-Aldrich), 1 mg/ml Hyaluronidase

(Roshe lifesciences) and 100 μg/ml DNase I (Sigma-Aldrich), and finally filtrated through 100 μm Nylon Mesh Filter. 5-10 x 10^6 cells were incubated with monensin (2 μM) for 5 h and stained with mouse CD45 (30-F11), CD90.2 (53-2.1), γδTCR (GL3), Lineage Antibody Cocktail from BD, Ly-6G/Ly-6C (RB6-8C5), TCRβ (H57-597), TCR Vγ1.1(2.11), Vγ2 (UC-10A6), Vγ3 (536) from BioLegend. Dead cells were stained with Fixable Viability Dye eFluor®780 (eBioscience). Isotype-matched control antibodies (BD) were used as controls. For intracellular staining, 25 ng/ml PMA and 1 ng/ml ionomycin was added into cell suspension and incubated for 2 h at 37°C followed with Brefeldin A Solution (BD) for 2 h at 37°C. Cells were first stained with cell surface markers and Fixable Viability Dye eFluor®780, and then fixed, permeabilized, and stained with anti-IL-17F (eBio18F10) and anti-IL-17A antibodies (eBio17B7) (eBioscience). Isotype-matched control antibodies (BD) were used as controls.

Antimicrobial Activity of Neutrophils

Neutrophils were isolated from wild-type and *Il17af*$^{-/-}$ mice described previously (Kim and Dinauer, 2001). 5 x 10^6 /ml neutrophils were stimulated with 20 nM PMA in RPMI medium for 4 h at 37°C in a 5% CO2 atmosphere. Then, *C. albicans* (SC5314) in yeast-form was added at a multiplicity of infection (MOI) of 0.02 and incubated with or without 100 ng/ml recombinant IL-17A (PeproTech) overnight at 37°C or 30°C in a 5% CO2 atmosphere. Whole samples were homogenized, plated on PDA plates with chloramphenicol, and incubated at 30°C to determine CFU. ROS production from neutrophils was measured by The ROS-Glo™ Assay (Promega).

Live Imaging of Hyphal Growth of *C. albicans*

C. albicans (SC5314) in yeast-form was incubated in RPMI medium at 37°C in a 5% CO2 atmosphere for 4 hrs to induce hyphal growth. Neutrophils from wild-type and *Il17af*$^{-/-}$ mice were pre-stimulated with 20 nM PMA in RPMI medium for 4 h at 37°C in a 5% CO2 atmosphere. Then, *C. albicans* in hyphal-form was added at a MOI of 0.02 into neutrophil culture and incubated overnight at 37°C. Pictures were taken every 10 min by Cytation 3 Cell Imaging Multi-Mode Reader (BioTek) and the live imaging movie were made by iMovie software (Apple).

Neutrophil Depletion in ECC

Mice were administrated with 500 µg/200 µl of anti Ly-6G antibody (1A8, Bio X cell) or 500 µg/200 µl of isotype IgG antibody (Bio X cell) intraperitoneally at day -1, 1, 3 and 5 in the ECC model.

General Experimental Design and Statistical Analysis

For animal experiments, a sample size of 3 to 10 mice per group was used on the basis of past experience in generating statistical significance. Prism software (GraphPad) was used for statistical analyses. The two-tailed Mann-Whitney test was used. P values of less than 0.05 were considered significant.

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FIGURES

Figure 1. Protective immunity against epicutaneous candidiasis requires IL-17A and F. (A) The mouse skin was colonized epicutaneously with 10^7 colony-forming units (CFU) of *C. albicans* using a gauze patch for 2 or 7 days. **(B)** Skin manifestations and histopathology of wild-type (WT), *Il17a*$^{-/-}$*, Il17f*$^{-/-}$ and *Il17af*$^{-/-}$ mice colonized with *C. albicans* or treated with PBS for 7 days. Skin sections were stained with haematoxylin and eosin (HE) and periodic acid-Schiff (PAS). Scale bar, 50 µm. Yellow arrow heads indicate *C. albicans* yeast and hyphae. Inset shows high-power image with hypha growth in the cornified layer of the epidermis. Representative of at least 5 mice per group. Right panel shows the number of inflammatory cells in skin of indicated mice colonized with *C. albicans* or treated with PBS. Results are depicted as the number of inflammatory cells per high-power field (HPF). **(C)** *C. albicans* CFU in the skin of wild-type, *Il17a*$^{-/-}$, *Il17f*$^{-/-}$ and *Il17af*$^{-/-}$ mice at day 2 and day 7 after inoculation. (**D**) *Il17a* and *Il17f* mRNA expression in skin of wild-type, *Il17a*$^{-/-}$*, Il17f*$^{-/-}$ and *Il17af*$^{-/-}$ mice colonized with *C. albicans* or treated with PBS for 2 days. **(E)** IL-17A and IL-17F levels in skin of WT, *Il17a*$^{-/-}$*, Il17f*$^{-/-}$ and *Il17af*$^{-/-}$ mice colonized with *C. albicans* or treated with PBS for 2 days. Dots represent individual mice. Error bars represent mean ± SEM. NS; no significant, *p<0.05, **p<0.01, ***p<0.001. Mann-Whitney test.

Figure 2. IL-17A and IL-17F are produced by γδ T cell and CD90$^+$ ILCs but not Gr-1$^+$ neutrophils in *C. albicans*-infected skin. (A, B) Total skin cell suspensions from pooled skin (n=2 or 3) of IL-17A-eGFP reporter mice and wild-type (WT) mice were stained for the indicated markers and analyzed by flow cytometry gated live/dead excluded, CD45$^+$ and eGFP$^+$. **(A)** Expression of CD90, Lineage, TCRβ and TCRγ was

used to identify ILC3, αβ T cells and γδ T cells. Right graph shows the percentage of IL-17A-eGFP^{+}cells in CD45^{+} population from the skin of indicated mice colonized with *C. albicans* or treated with PBS. **(B)** Expression of Gr-1 was used to identify neutrophils. **(C, D)** Total skin cell suspensions from pooled skin (n=3) of wild-type mice were stained for the indicated markers and analyzed by flow cytometry gated live/dead excluded, CD45^{+}. (**C**) Expression of IL-17F in CD90^{+} and Lineage^{+} cells. Right graph shows the percentage of IL-17F^{+} cells in CD45^{+} population from the skin of wild-type mice colonized with *C. albicans* or treated with PBS. **(D)** Expression of IL-17F in Gr-1^{+} cells. Error bars represent mean ± SEM. NS; no significant, *p<0.05, **p<0.01, ***p<0.001. Mann-Whitney test.

Figure 3. CD90^{+} ILCs are key players in host defense against ECC. (A) Skin manifestations and histology of wild-type (WT), *Rag2*$^{-/-}$, *Rag2Il2rg*$^{-/-}$ and *Il17af*$^{-/-}$ mice treated with PBS or colonized with *C. albicans* 10^7 CFU epicutaneously for 7 days. Skin sections were stained with HE and PAS. Scale bar, 50 μm. Yellow arrow heads indicate *C. albicans* yeast and hypha. Representative of at least 5 mice per group. **(B)** *C. albicans* CFU from the skin of wild-type, *Rag2*$^{-/-}$, *Il17af*$^{-/-}$ and *Rag2Il2rg*$^{-/-}$ mice at day 2 and day 7 after colonization. **(C)** *Il17a* and *Il17f* mRNA expression in skin of wild-type, *Rag2*$^{-/-}$, *Il17af*$^{-/-}$ and *Rag2Il2rg*$^{-/-}$ mice colonized with *C. albicans* or treated with PBS for 2 days. **(D)** IL-17A and IL-17F levels in skin of wild-type, *Rag2*$^{-/-}$, *Il17af*$^{-/-}$ and *Rag2Il2rg*$^{-/-}$ mice colonized with *C. albicans* or treated with PBS for 2 days. Dots represent individual mice. Error bars represent mean ± SEM. NS; no significant, *p<0.05, **p<0.01, ***p<0.001. Mann-Whitney test.

Figure 4. TLR2, MyD88, Dectin-1, Dectin-2, FcRγ and Card9 are dispensable for *C. albicans* clearance in ECC model. (A) Skin manifestations and each histopathology of wild-type (WT), *Tlr2*$^{-/-}$, *Myd88*$^{-/-}$, *Clec7aClec4n*$^{-/-}$, *Fcer1g*$^{-/-}$, *Card9*$^{-/-}$ and *Il17af*$^{-/-}$ mice colonized with *C. albicans* or treated with PBS at 7 days. Skin sections were stained with HE and PAS. Scale bar, 50 µm. Yellow arrow heads indicate *C. albicans* yeast and hypha. Inset shows high-power image with hypha growth in the cornified layer of the epidermis. Representative of at least 5 mice per group. **(B)** *C. albicans* CFU from the skin of wild-type, *Tlr2*$^{-/-}$, *Clec7aClec4n*$^{-/-}$, *Fcer1g*$^{-/-}$, *Card9*$^{-/-}$ and *Il17af*$^{-/-}$mice at day 7 after colonization. Dots represent individual mice. NS; no significant, ***p<0.001. Mann-Whitney test.

Figure 5. IL-17 plays a pivotal role in host defense against ECC by recruiting and activating neutrophils. (A) *S100A8* and *S100A9* mRNA expression in skin of wild-type (WT), *Il17a*$^{-/-}$, *Il17f*$^{-/-}$ and *Il17af*$^{-/-}$ mice colonized with *C. albicans* or treated with PBS for 2 days. **(B)** Immunofluorescence staining of S100A9, Ly-6G and MPO in wild-type and *Il17af*$^{-/-}$ mice colonized with *C. albicans* or treated with PBS for 2 days. Representative of at least 3-4 mice per group. **(C)** *C. albicans* co-cultured with or without neutrophils isolated from wild-type and *Il17af*$^{-/-}$ mice at a multiplicity of infection (MOI) of 0.02 for 24 hours at 37°C. **(D)** *C. albicans* CFU after incubation with neutrophils from wild-type and *Il17af*$^{-/-}$ mice for 24 hours.

Error bars represent mean ± SEM. NS; no significant, *p<0.05, **p<0.01. Mann-Whitney test.

Figure 6. Neutrophil-depleted mice are impaired in the clearance of *C. albicans* in ECC. (A) Wild-type (WT) mice were colonized epicutaneously with 10^7 colony-forming units (CFU) of *C. albicans* using a gauze patch for 7 days. Ly-6G antibody- or isotype IgG-administration were performed at day -1, 1, 3 and 5 intraperitoneally. **(B)** Skin phenotype and histology of *Il17af*$^{-/-}$ mice and wild-type mice treated with Ly-6G antibody or isotype control IgG colonized with *C. albicans* or treated with PBS epicutaneously for 7 days. Results are depicted as the number of inflammatory cells per HPF. Yellow arrow heads indicate *C. albicans* yeast and hypha. **(C)** *C. albicans* CFU from the skin of wild-type mice treated with Ly-6G antibody or isotype IgG at day 7 after inoculation.

SUPPLEMENTAL INFORMATION

Supplementary Figure 1. Innate immune response to epicutaneous candidiasis requires IL-17 production. Skin phenotype and histopathology of wild-type (WT), *Il17a*$^{-/-}$, *Il17f*$^{-/-}$ and *Il17af*$^{-/-}$ mice inoculated with *C. albicans* or treated with PBS for 2 days. Skin sections were stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS). Scale bar, 50 µm. Yellow arrow heads indicate *C. albicans* yeast and hypha. Representative of at least 5 mice per group.

Supplementary Figure 2. Inflammatory cytokine and chemokine amounts in skin of wild-type, *Il17a*$^{-/-}$, *Il17f*$^{-/-}$ and *Il17af*$^{-/-}$ mice inoculated with *C. albicans* or treated with PBS for 2 days. Dots represent individual mice. Error bars represent mean ± SEM. NS; no significant, *p<0.05, **p<0.01, ***p<0.001. Mann-Whitney test.

Supplementary Figure 3. Taxonomic composition of skin and fecal bacteria of wild-type and *Il17af*$^{-/-}$ mice. The dorsal skin of mice was shaved with electric clippers and hair removal agent 4 days prior to the sample collection.

Supplementary Figure 4. IL-17 expression in Vγ TCR segments of Vγ1.1^{+}, Vγ2^{+} or Vγ3^{+} populations in ECC. Wild-type (WT) and IL-17A-eGFP reporter mice were colonized with *C. albicans* for 2 days. **(A)** Total skin single cell suspensions from pooled skin (n=2 or 3) of WT and IL-17A-eGFP reporter mice were stained for the indicated markers and analyzed by flow cytometry gated live/dead excluded, CD45^{+} and eGFP^{+}. **(B)** Total skin single cell suspensions from pooled skin (n=3) of WT mice were stained for IL-17F intracellularly and analyzed by flow cytometry gated live/dead

excluded, $CD45^+$. The percentage in red color indicates IL-17F positive population.

Supplementary Figure 5. *C. albicans* infected skin from *Rag2Il2rg*$^{-/-}$ mice has neither IL-17A nor IL-17F positive cells. Wild-type (WT) and *Rag2Il2rg*$^{-/-}$ mice were inoculated epicutaneously with *C. albicans* or treated with PBS for 2 days. Total skin cell suspensions from pooled skin (n=3) were stained with IL-17A and IL-17F intracellularly and analyzed by flow cytometry gated live/dead excluded and $CD45^+$.

Supplementary Figure 6. IL-17 does not affect Dectin-1, S100A9 mRNA expression, NETs release and ROS production in neutrophils *in vitro*. (A) *C. albicans* co-cultured with or without neutrophils isolated from wild-type (WT) mice at a multiplicity of infection (MOI) of 0.02 for 24 hours at 30°C. *C. albicans* CFU after incubation with neutrophils from WT mice for 24 hours. **(B)** Dectin-1 (*Clec7a*), *S100a9* mRNA expression in neutrophils isolated from WT, *Il17af*$^{-/-}$ and *Clec7aClec4n*$^{-/-}$ mice. S100A9 protein expression was examined by immunoblot analysis with WT and *Il17af*$^{-/-}$ neutrophils. **(C)** *C. albicans* co-cultured with or without neutrophils isolated from WT and *Il17af*$^{-/-}$ mice at a multiplicity of infection (MOI) of 0.02 for 24 hours. NETs release was detected by the staining of Hoechst 33342. **(D)** Reactive oxygen species (ROS) production from 2 x 10^{6} neutrophils from wild-type and *Il17af*$^{-/-}$ mice stimulated with indicated CFU/ml of *C. albicans* with or without rIL-17A (10 ng/ml) for 1hrs. RLU = Relative light unit. Error bars represent mean ± SEM. NS; no significant

Supplemantary Movie 1. IL-17 is important for migration, sensing and phagocytosis of hyphal forms in neutrophils. Red circle indicated hyphal form of *C. albicans.* One second indicate 10 min of co-culture.

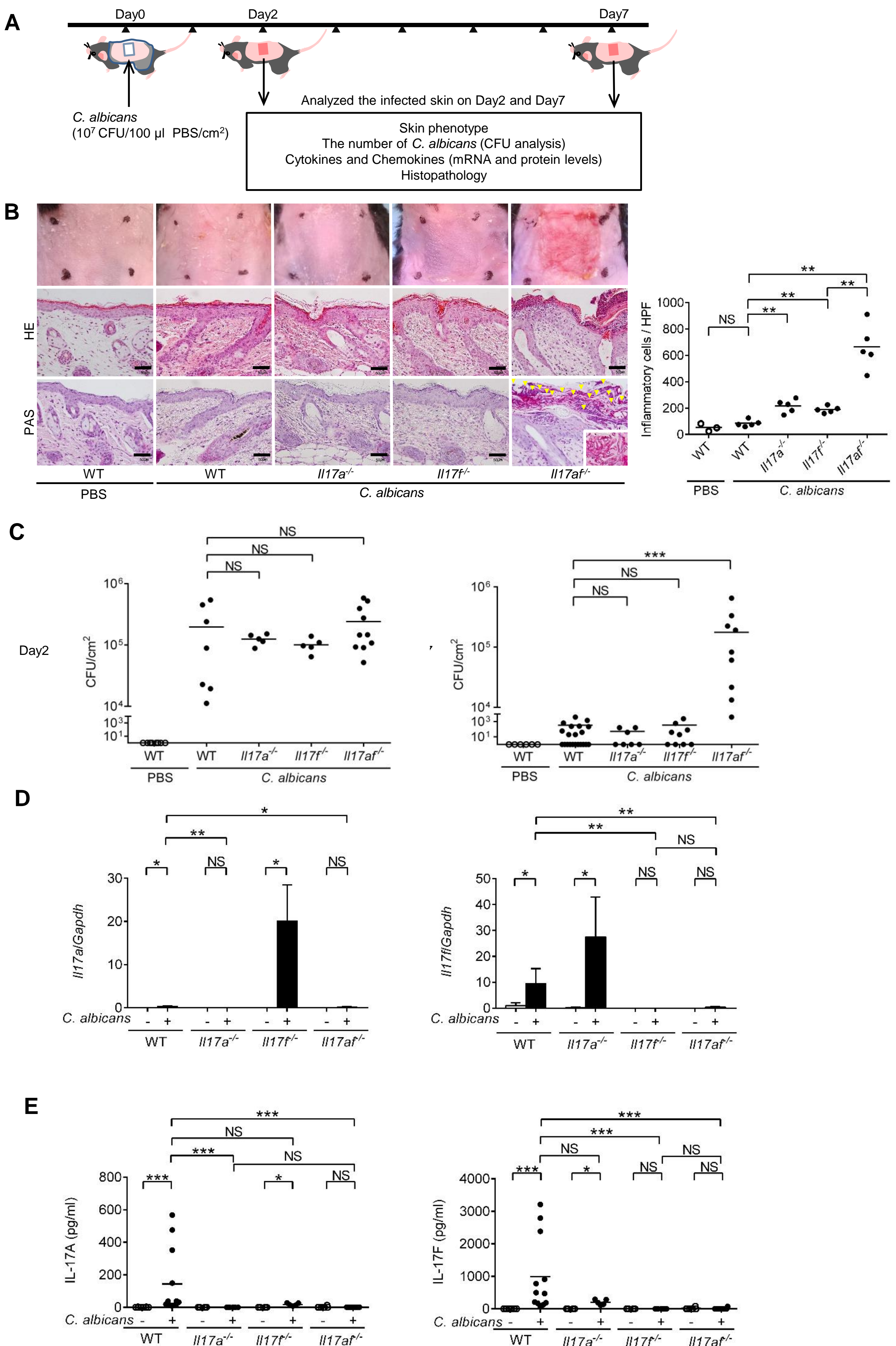
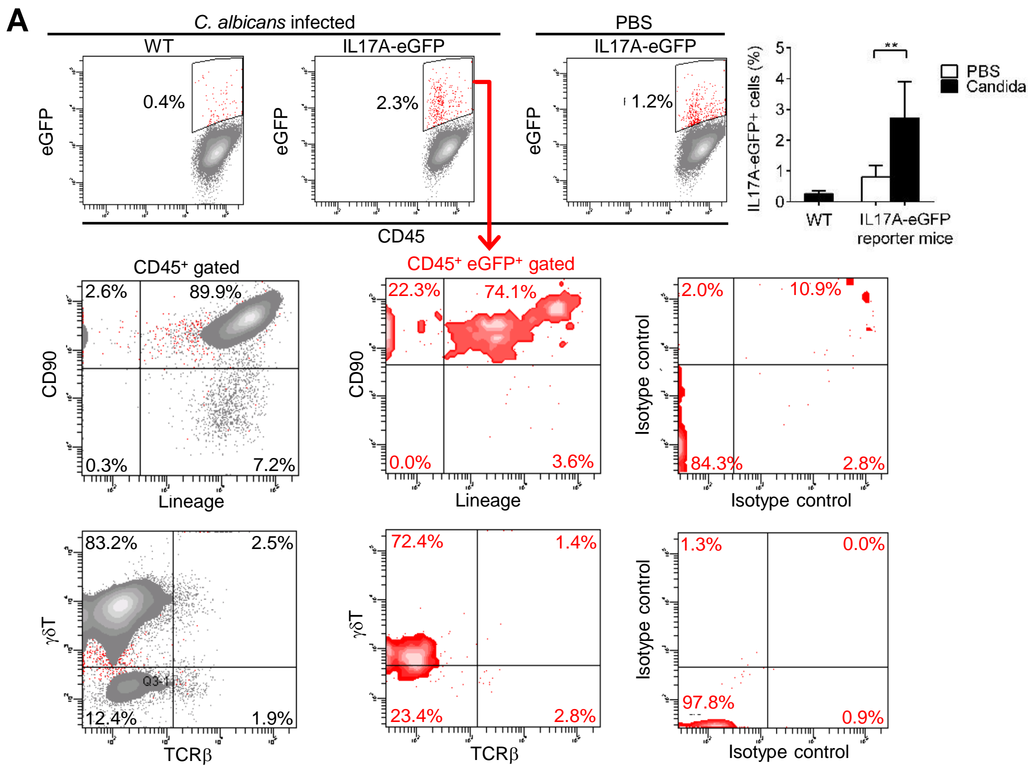
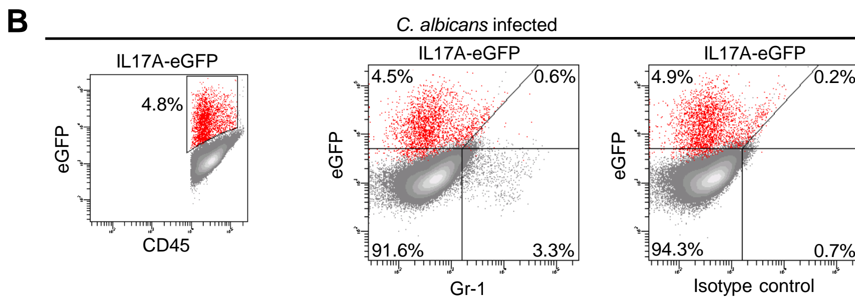
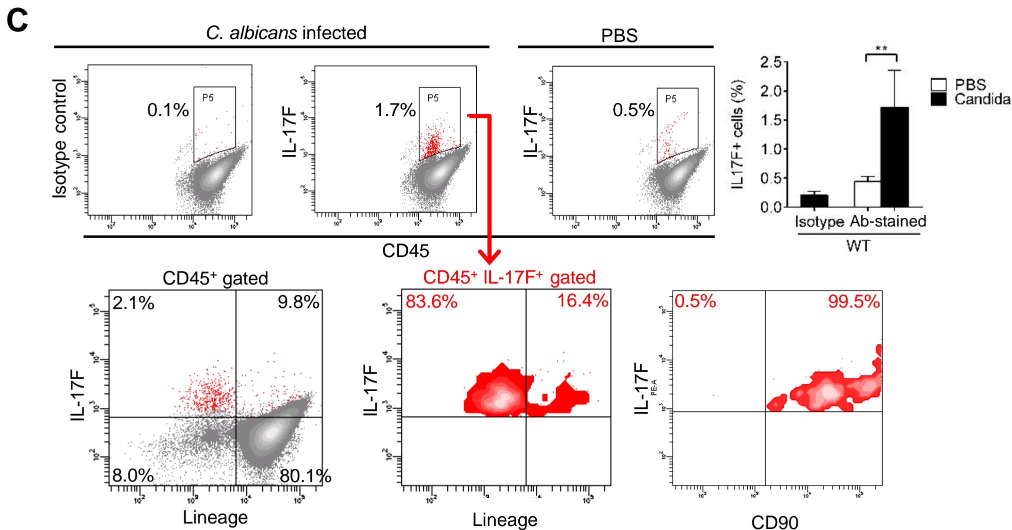


Figure 1







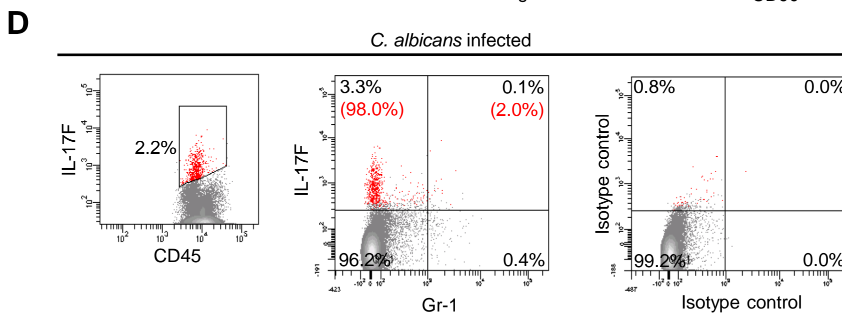
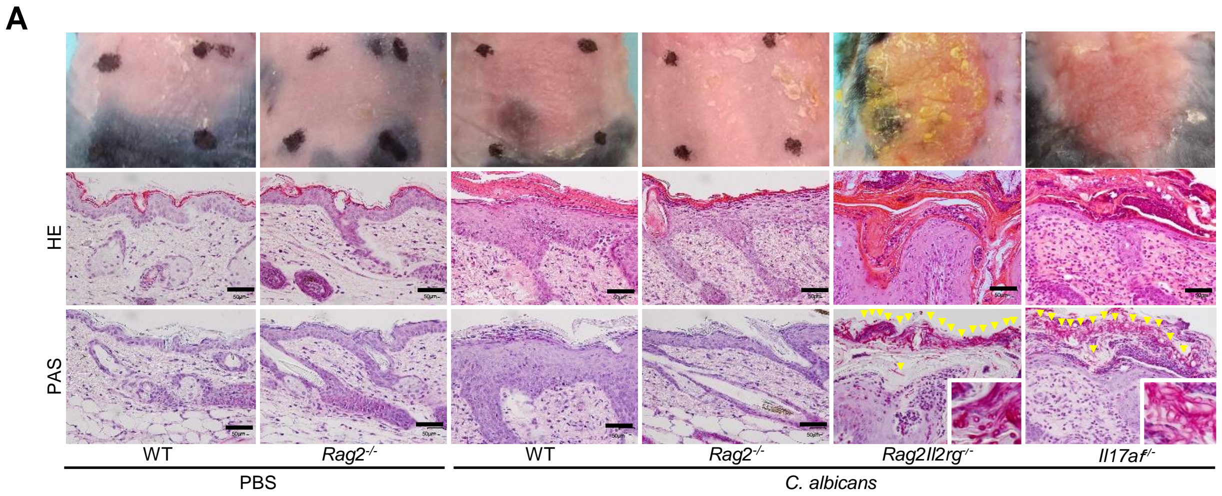
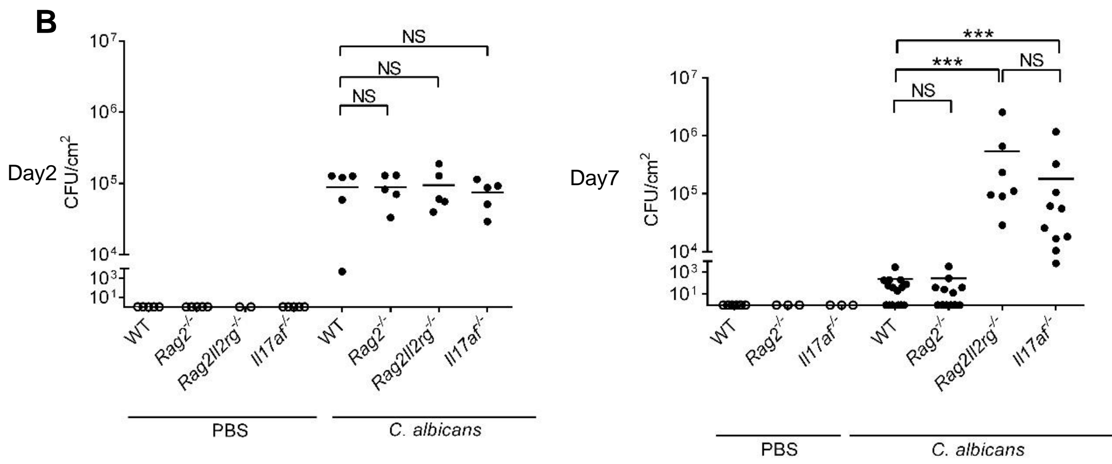
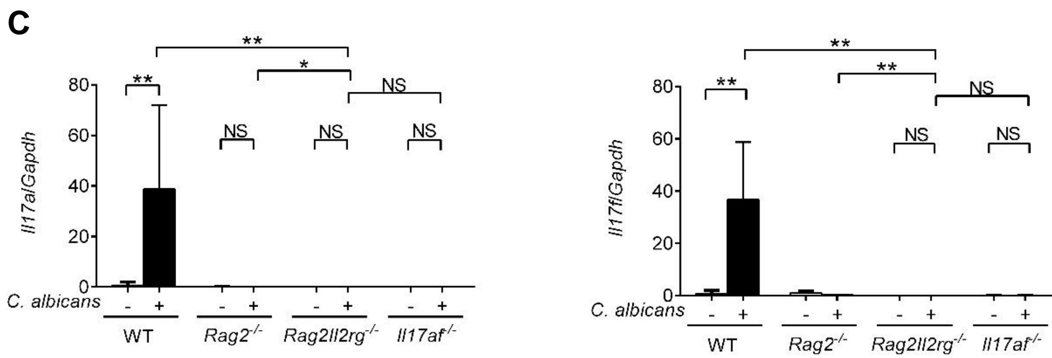


Figure 2







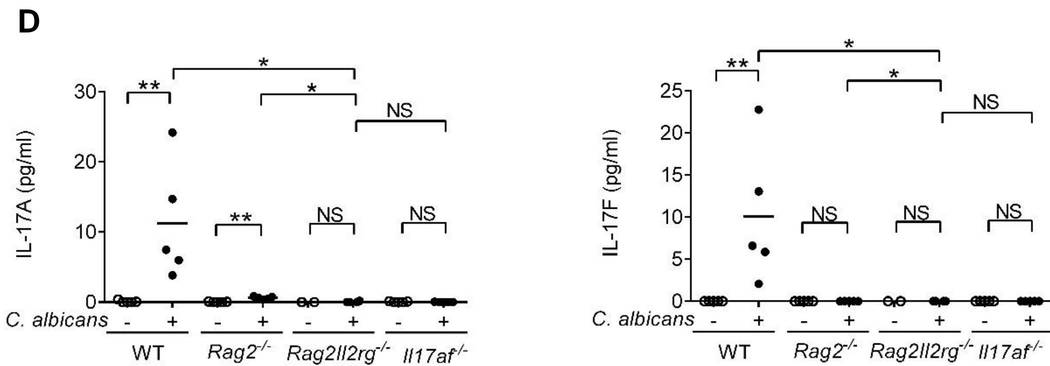
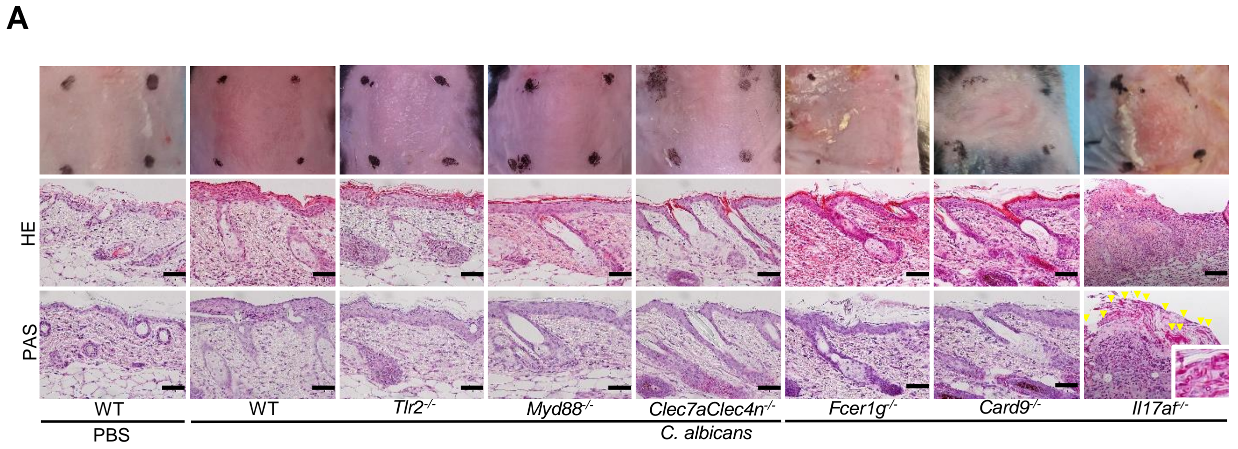


Figure 3

A



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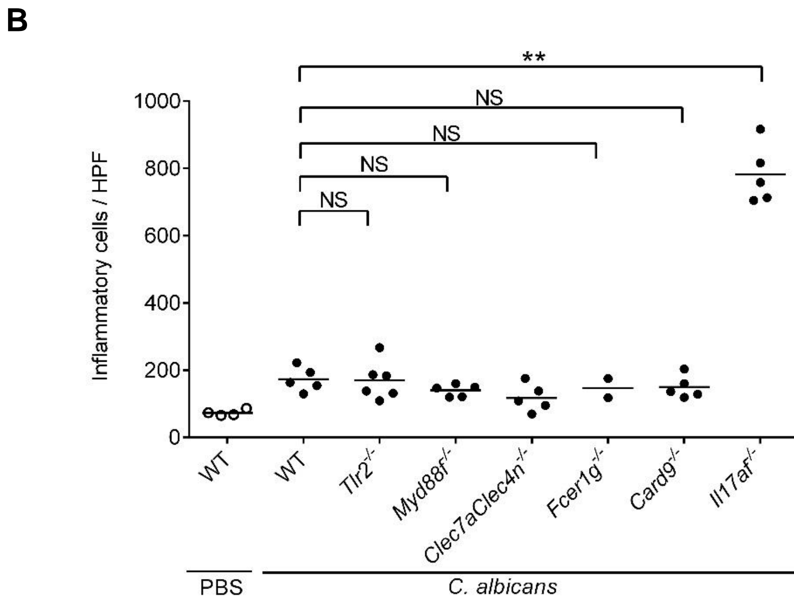

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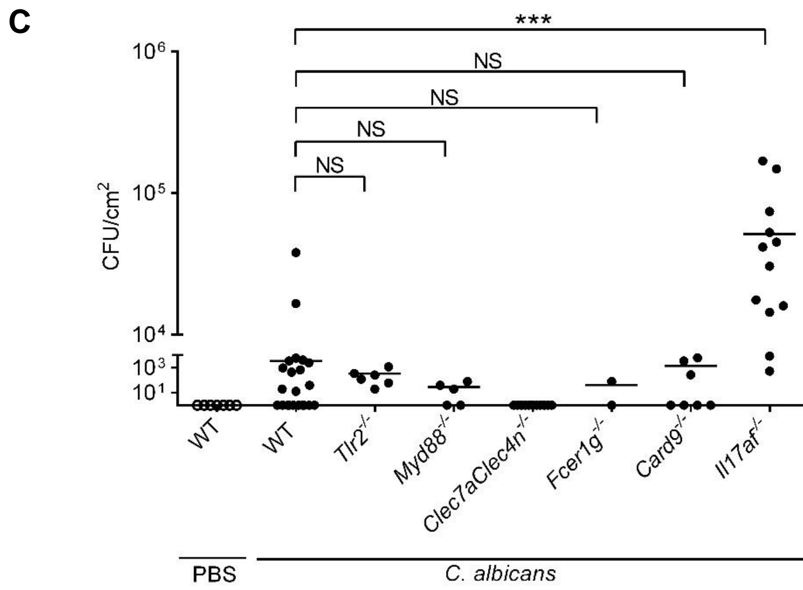


Figure 4

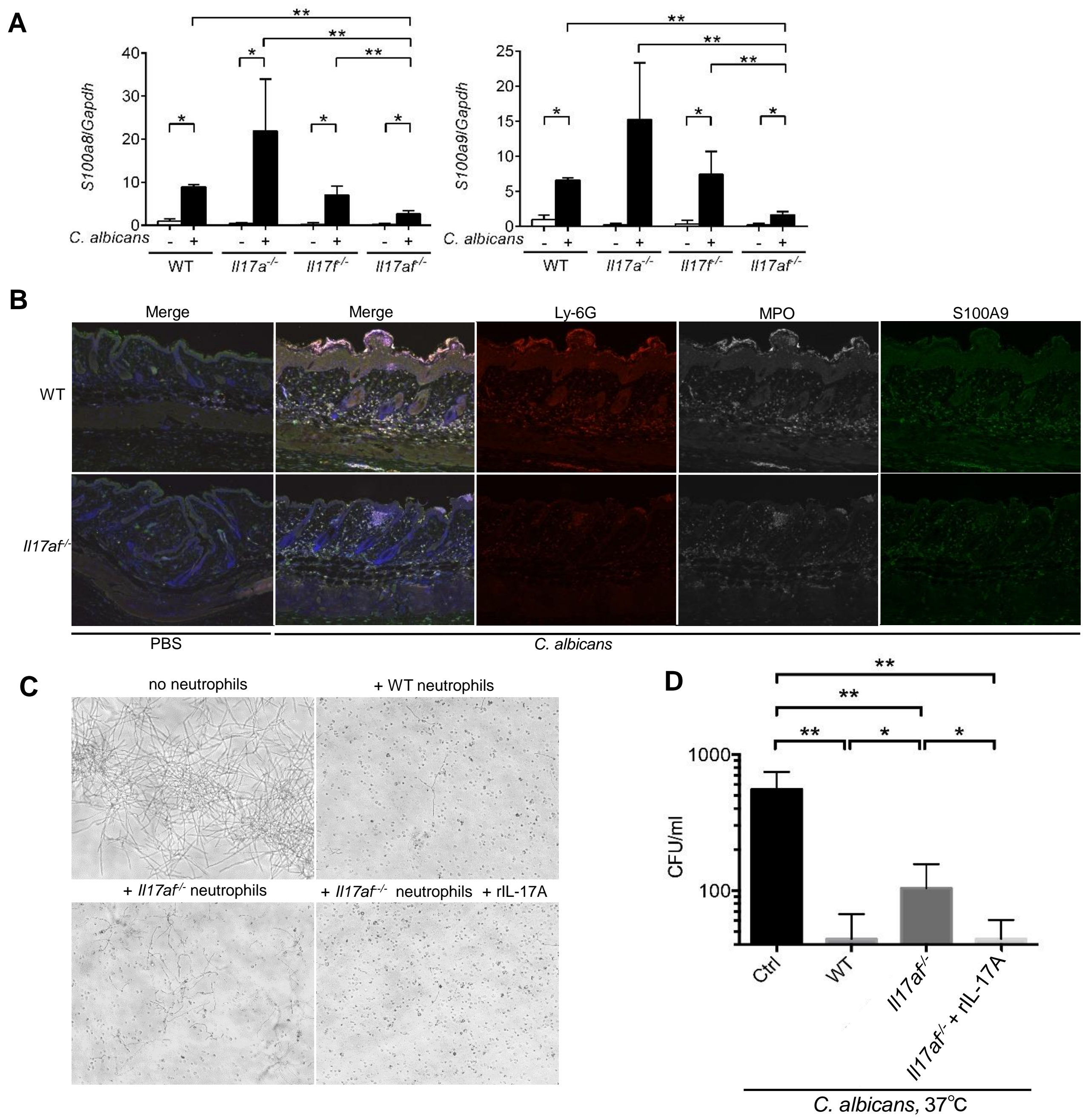
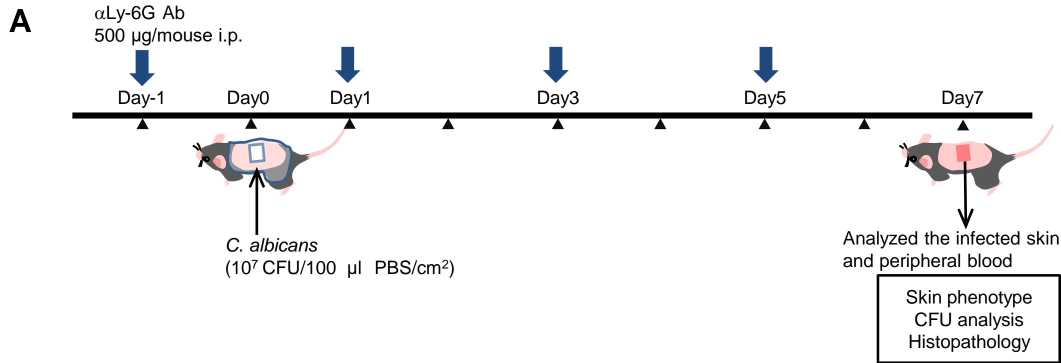
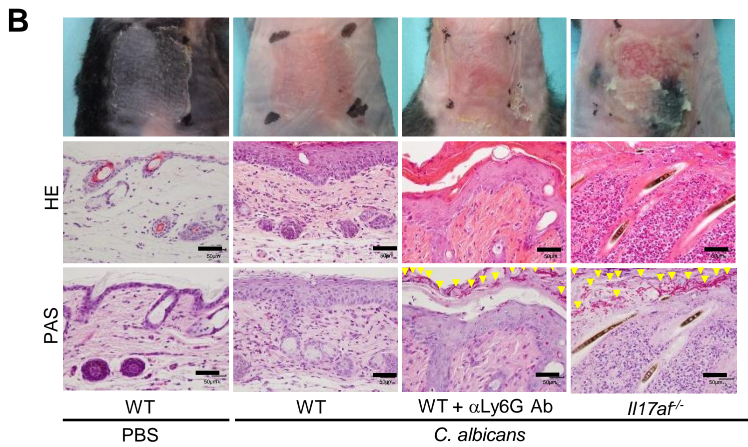


Figure 5





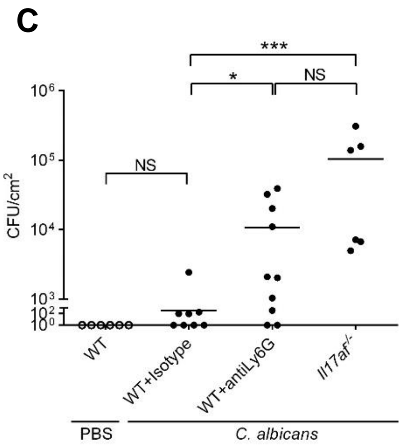
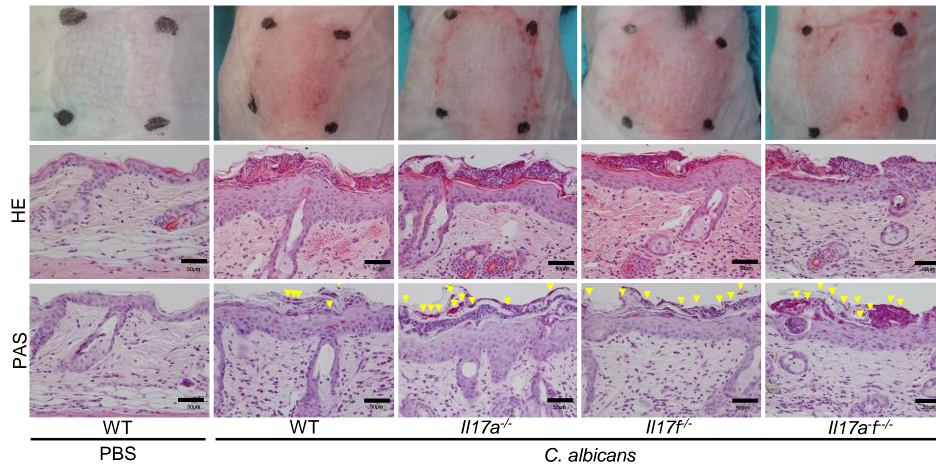


Figure 6



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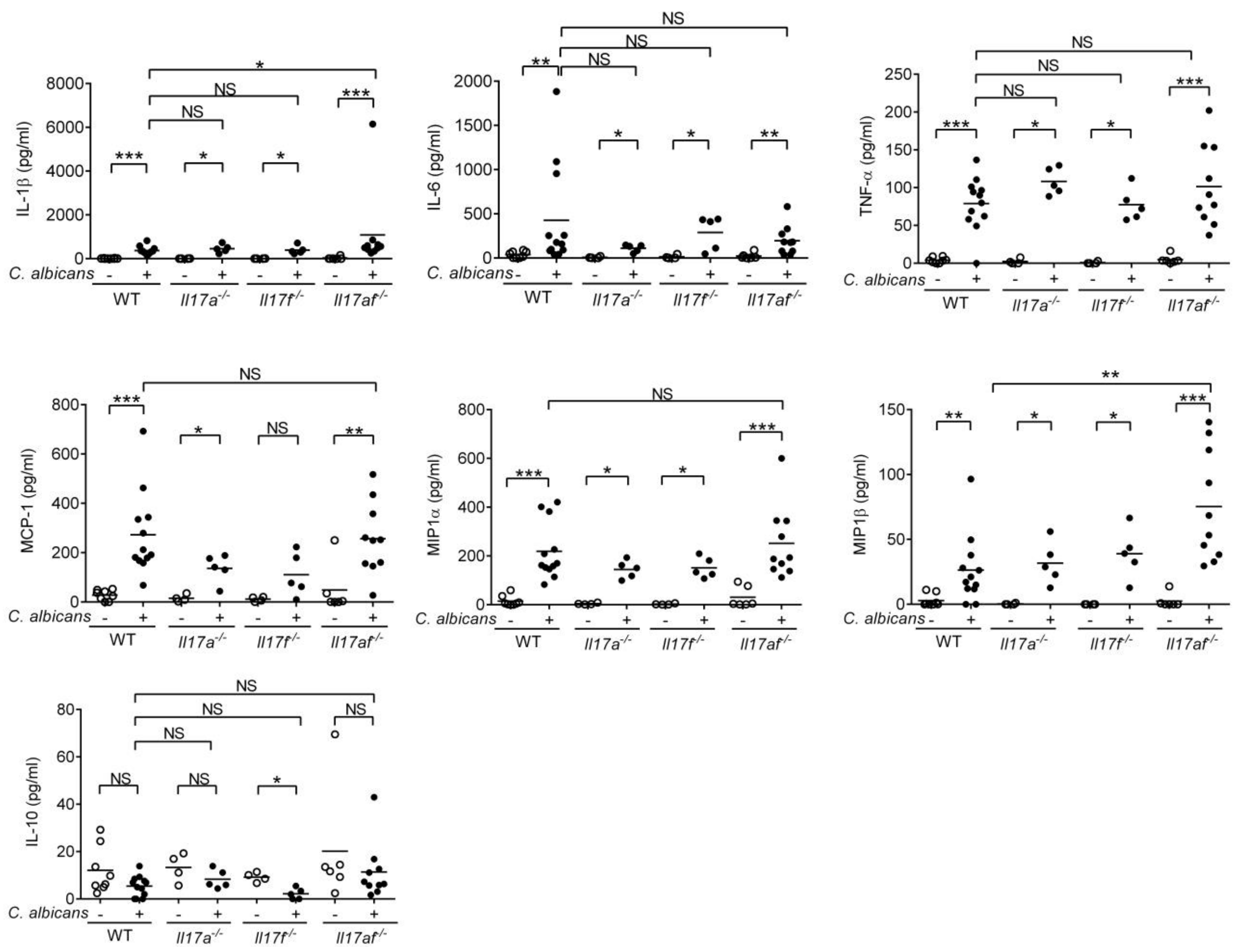

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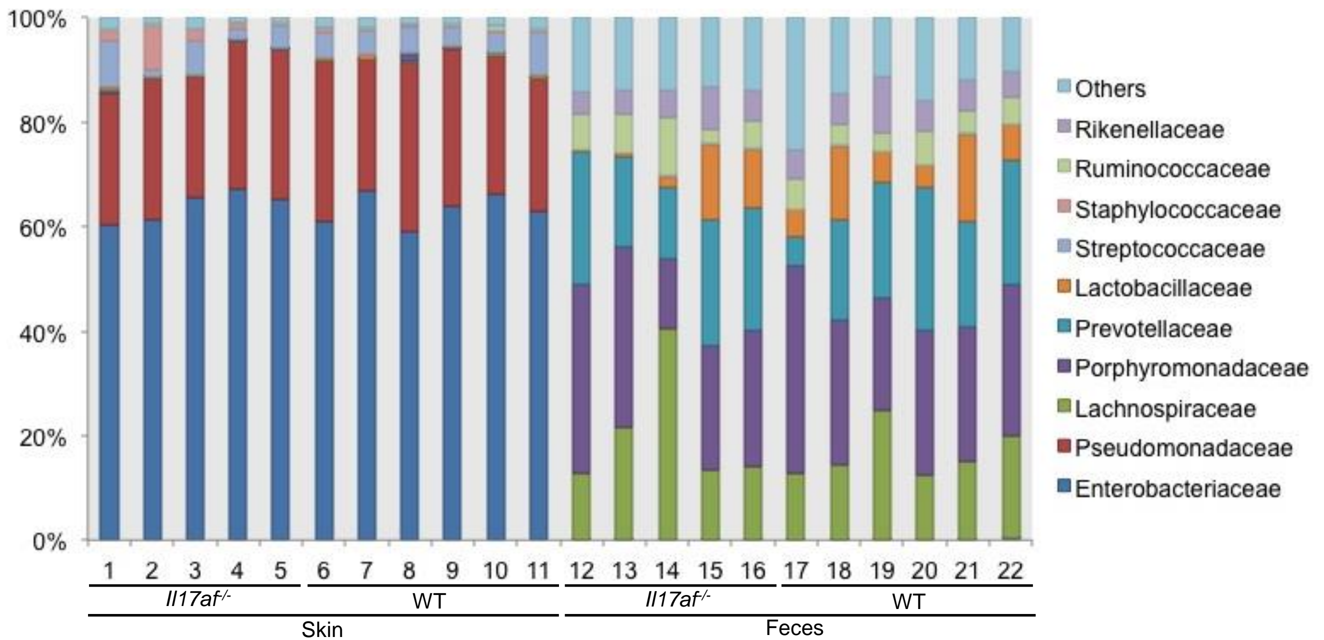

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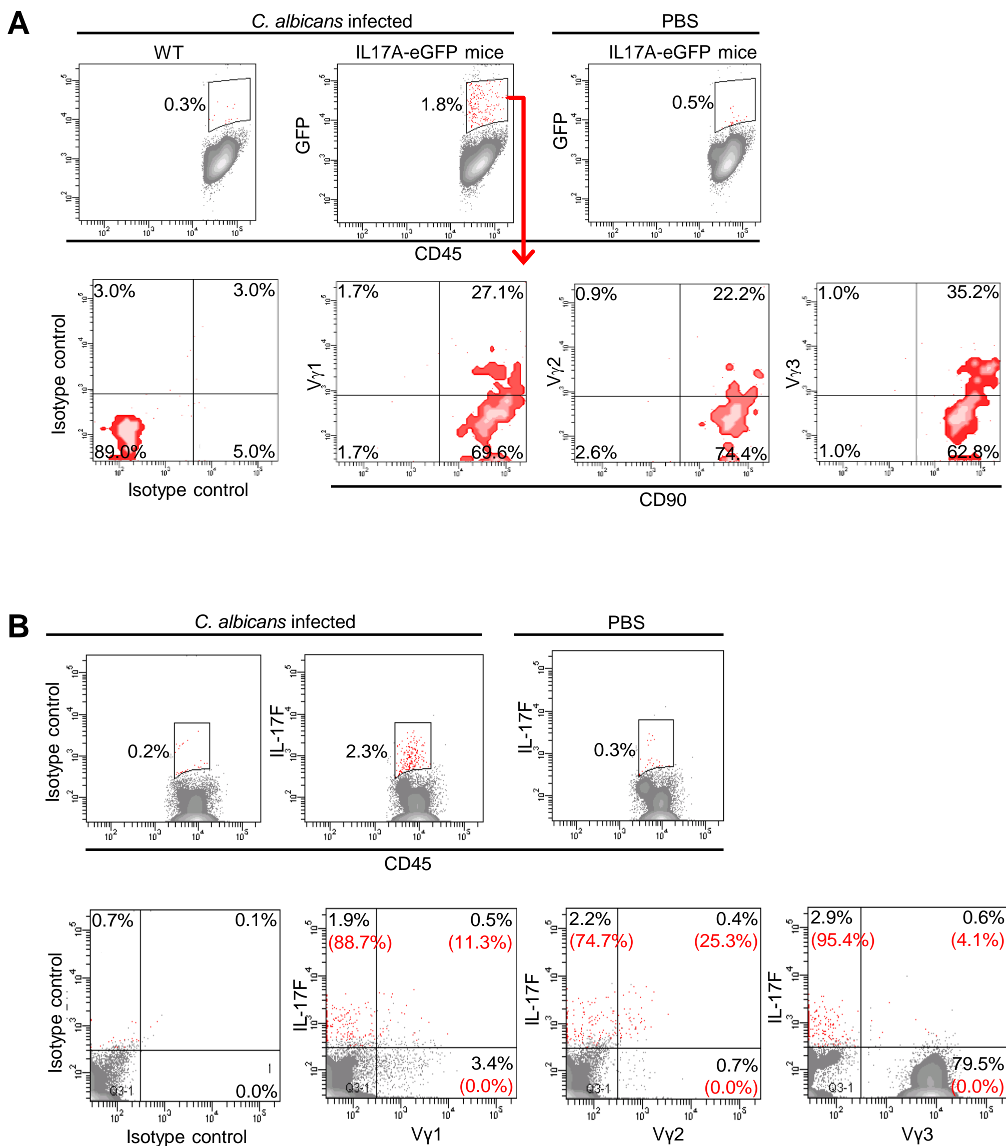
Supplementary Figure 3



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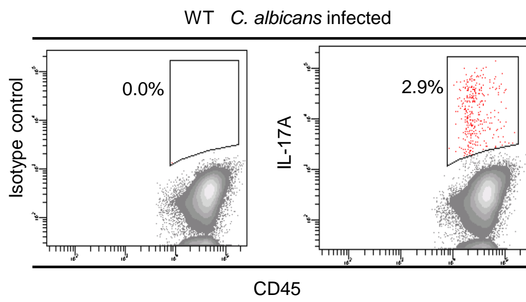
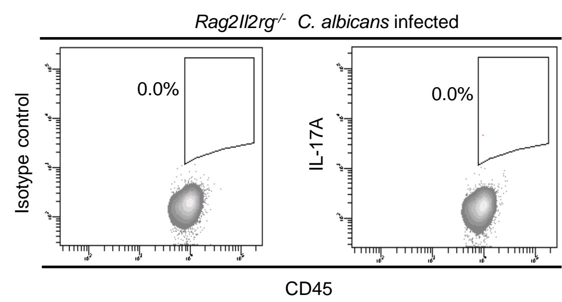
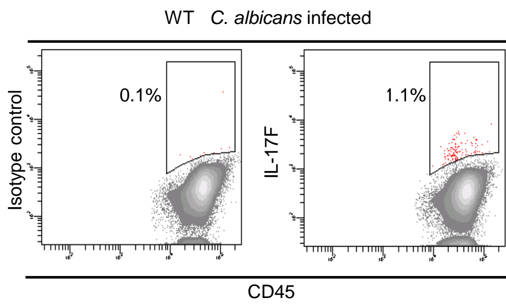
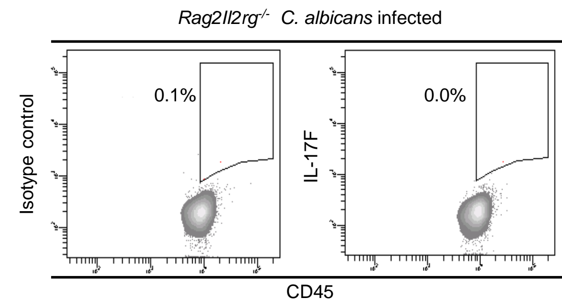
Supplementary Figure 4



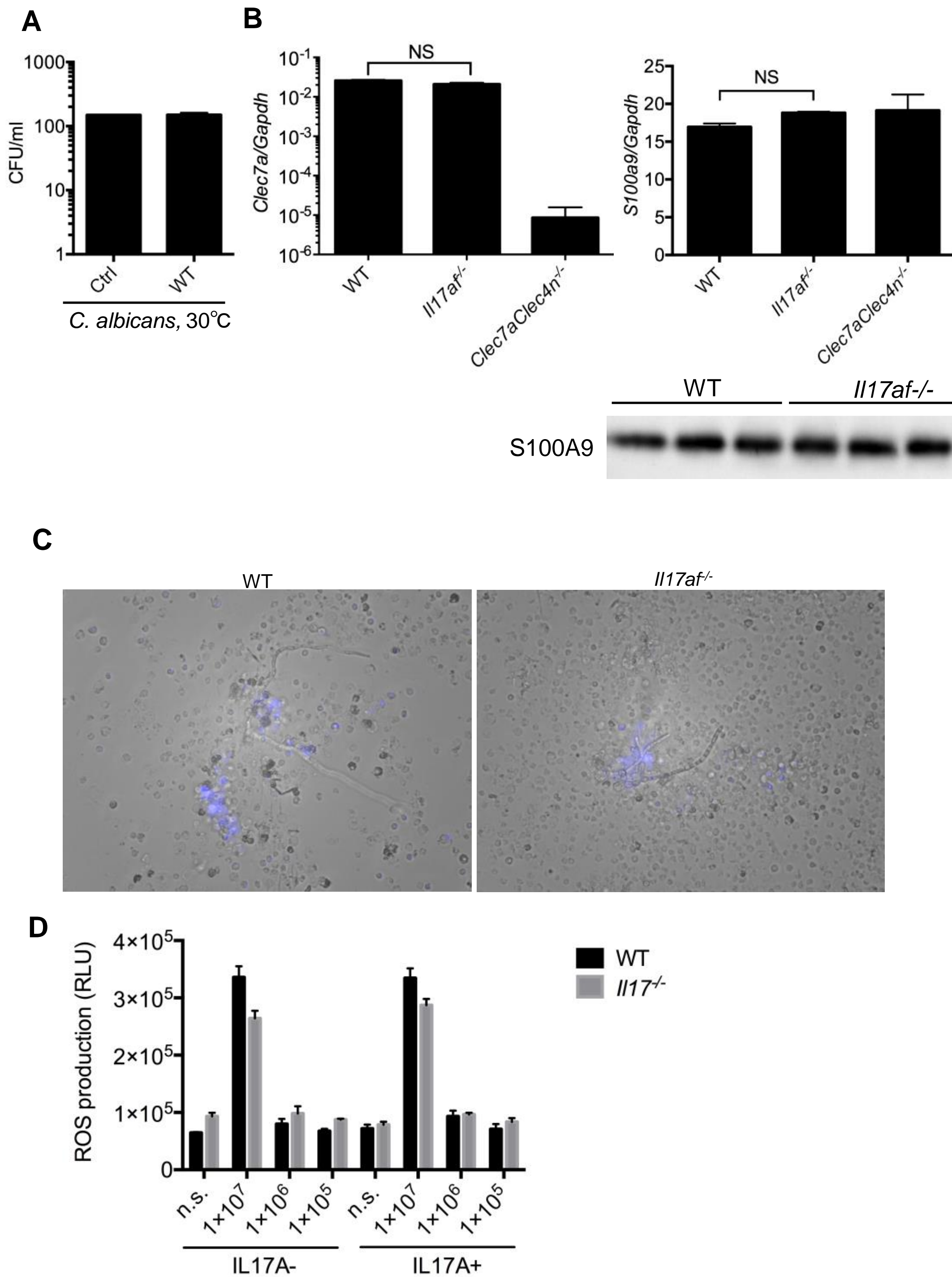






Supplementary Figure 5. *C. albicans* infected skin from *Rag2Il2rg*$^{-/-}$ mice has neither IL-17A nor IL-17F positive cells. Wild-type (WT) and *Rag2Il2rg*$^{-/-}$ mice were inoculated epicutaneously with *C. albicans* or treated with PBS for 2 days. Total skin cell suspensions from pooled skin (n=3) were stained with IL-17A and IL-17F intracellularly and analyzed by flow cytometry gated live/dead excluded and CD45^{+}.

Supplementary Figure 5



Supplementary Figure 6. IL-17 does not affect Dectin-1, S100A9 mRNA expression, NETs release and ROS production in neutrophils *in vitro*. (A) *C. albicans* co-cultured with or without neutrophils isolated from wild-type (WT) mice at a multiplicity of infection (MOI) of 0.02 for 24 hours at 30° C . *C. albicans* CFU after incubation with neutrophils from WT mice for 24 hours. **(B)** Dectin-1 (*Clec7a*), *S100a9* mRNA expression in neutrophils isolated from WT, *Il17af*$^{-/-}$ and *Clec7aClec4n*$^{-/-}$ mice. S100A9 protein expression was examined by immunoblot analysis with WT and *Il17af*$^{-/-}$ neutrophils. **(C)** *C. albicans* co-cultured with or without neutrophils isolated from WT and *Il17af*$^{-/-}$ mice at a MOI of 0.02 for 24 hours. NETs release was detected by the staining of Hoechst 33342. (D) Reactive oxygen species (ROS) production from 2 x 10^6 neutrophils from WT and *Il17af*$^{-/-}$ mice stimulated with indicated CFU/ml of *C. albicans* with or without rIL-17A (10 ng/ml) for 1hr. RLU = Relative light unit. Error bars represent mean ± SEM. NS; no significant

Supplementary Figure 6

（平成27年12月15日Cell投稿中）