Supplementary Information for

Immunity to commensal skin fungi promotes psoriasiform skin inflammation

Charlotte Hurabielle[a,b,c,1], Verena M. Link[a,1], Nicolas Bouladoux[a,d], Seong-Ji Han[a], Eric Dean Merrill[a,e], Yaima L. Lightfoot[f,g], Nickie Seto[f], Christopher K. E. Bleck[h], Margery Smelkinson[i], Oliver J. Harrison[a,j], Jonathan L. Linehan[a,k], Samira Tamoutounour[a,l], Michail S. Lionakis[m], Mariana J. Kaplan[f], Saeko Nakajima[a,n,2] and Yasmine Belkaid[a,d,2]

[a] Metaorganism Immunity Section, Laboratory of Immune System Biology, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD 20892.
[b] Inserm Unit 976, Hôpital Saint-Louis, 75010 Paris, France.
[c] San Francisco Internal Medicine, University of California, San Francisco, CA 94143.
[d] Microbiome Program, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD, 20892.
[e] San Francisco Dermatology, University of California, San Francisco, CA 94115.
[f] Systemic Autoimmunity Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, NIH, Bethesda, MD 20892.
[g] Neurodegeneration Consortium, University of Texas MD Anderson Cancer Center, Houston, TX 77054.
[h] Electron Microscopy Core facility, National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892.
[I] Biological Imaging, Research Technology Branch, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD 20892.
[j] Immunology Program, Benaroya Research Institute, Seattle, WA 98101.
[k] Department of Cancer Immunology, Genentech, South San Francisco, CA 94080.
[l] Current affiliation: L'Oréal Advanced Research, L'Oréal Research and Innovation, 93600 Aulnay-sous-Bois, France.
[m] Fungal Pathogenesis Section, Laboratory of Clinical Immunology and Microbiology, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD 20892.
[n] Department of Dermatology, Kyoto University Graduate School of Medicine, Sakyo, 606-8507 Kyoto, Japan.

[1]Both authors contributed equally

[2]co-corresponding authors; Yasmine Belkaid (ybelkaid@niaid.nih.gov) and Saeko Nakajima (fujiko@kuhp.kyoto-u.ac.jp)

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Supplementary Methods

Mice.

C57BL/6 specific pathogen free (SPF) mice were purchased from Taconic Farms. Germ-free C57BL/6 mice were bred and maintained at the NIAID Microbiome Program gnotobiotic animal facility. B6.SJL Cd45a(Ly5a)/Nai (B6.SJL), C57BL/6NTac-[KO]Abb/B2m (*MHCII*$^{-/-}$), B6.129P2(C)-*Baft3tm1Kmm*/J (*Batf3*$^{-/-}$), B6.SJL-Cd45a(Ly5a)Nai-[KO]RAG1 and C57BL/6-[TgH]EGFP:Foxp3 mice (FoxP3-GFP reporter mice) were obtained through the NIAID-Taconic exchange program. B6.FVB-Tg(CD207-Dta)312Dhka/J (Lan-DTA) mice were obtained from Dr. Kaplan (University of Pittsburgh). *Irf4*$^{fl/fl}$ x *Itgax*$^{cre+}$ mice were obtained by breeding B6.129S1-*Irf4*^{tm1Rdf}/J (*Irf4*$^{fl/fl}$) mice with B6.Cg-Tg(Itgax–cre)1-1Reiz/J (*Itgax–cre*) mice (both strains from The Jackson Laboratory). *Rorc*$^{fl/fl}$ *x Lck*$^{cre+}$ mice, a kind gift from Dr. Bosselut (NIH/NCI), were obtained by breeding B6(Cg)-Rorctm3Litt/J (*Rorc*$^{fl/fl}$) mice with B6.Cg-Tg(Lck-icre)3779Nik/J (*Lck*$^{cre+}$) mice (both strains from The Jackson Laboratory). B6.129S6-Card9tm1Xlin/J (*Card9*$^{-/-}$) mice and B6.129S6-Clec7atm1Gdb/J (*Clec7a*$^{-/-}$) mice were obtained from Dr. Lionakis (NIH/NIAID). All mice were bred and maintained under pathogen-free conditions at an American Association for the Accreditation of Laboratory Animal Care (AAALAC)-accredited animal facility at the NIAID and housed in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals. All experiments were performed at the NIAID under an animal study proposal approved by the NIAID Animal Care and Use Committee. Gender- and age-matched mice between 6 and 16 weeks of age were used for each experiment. When possible, preliminary experiments were performed to determine requirements for sample size, taking into account resources available and ethical, reductionist animal use. Exclusion criteria such as inadequate staining or low cell yield due to technical problems were predetermined. Animals were assigned randomly to experimental groups.

Topical association and intradermal infection of mice with fungi species.

Unless stated otherwise, a *Candida albicans* strain that was isolated from the ear pinnae of *Il22*$^{-/-}$ mice was used. Furthermore, the reference strain of *C. albicans* SC5314 (ATCC MYA-2876) and another *C. albicans* strain isolated from human psoriatic skin (referred to as human *C. albicans*) were used. Yeast locked *C. albicans* (*hgc1*∆) and its control strain *hgc1*∆+HGC1^{+} were gifted by Dr. Lionakis (NIH/NIAID). *C. albicans* strains were grown in either yeast extract peptone dextrose (YPD) medium or Tryptic Soy Broth (TSB) at 30°C with shaking for 18 h prior to association. *M. furfur* (ATCC 14521) and *T. mentagrophytes* (ATCC 18748) were purchased from

ATCC and cultured as recommended by ATCC. For topical association, mice were associated with fungi by applying fungal suspension (10^9 CFU/ml) using a sterile cotton swab. Application of fungal suspension was repeated every other day for 8 days. For intradermal infection, mice were injected with 10^7 CFU of *C. albicans.*

Tissue processing.

Mice were euthanized 14 days after the first topical application of *C. albicans* and/or the day after the last topical application of IMQ. Single cell suspension from the skin draining lymph nodes or the ear pinnae were isolated as previously described (1). In brief, skin draining lymph nodes were dissociated through 10 μm sterile filters. Ear pinnae were digested in RPMI containing 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and nonessential amino acids, 50 μM β-mercaptoethanol, 20 mM HEPES (HyClone), and 0.25 mg/ml Liberase TL (Roche Diagnostic Corp.), or 2.5 mg/ml collagenase D (Roche) and incubated for 1 h 45 min at 37°C and 5% CO2. Digested skin sheets were homogenized using a Medicon/Medimachine tissue homogenizer system (Becton Dickinson).

Flow cytometry.

Single-cell suspensions were first stained with the fluorochrome-conjugated antibodies against surface markers CD1d (clone 1B1), CD3ε (17A2), CD4 (RM4-5), CD8α (eBioH35-17.2), CD11b (M1/70), CD11c (N418), CD19 (eBio1D3), CD31 (MEC13.3), CD44 (IM7), CD45 (30-F11), CD45.1 (A20), CD45.2 (104), CD45R (RA3-6B2), CD49b (DX5), CD49f (eBioGoH3), CD64 (FA-11), CD90.2 (53-2.1), CD103 (2E7), CD117 (2B8), CD192 (CCR2, 475301), CD335 (Nkp46, 29A1.4), CD369 (Dectin-1, RH-1), FcεRI (MAR-1), Ly6C (HK1.4), Ly6G (1A8), MHC-II (M5/114.15.2), NK1.1 (PK136), Sca-1 (D7), Siglec-F (E50-2440), TCRβ (H57-597), and/or γδTCR (eBioGL3) for 20-30 min at 4°C in Phosphate Buffer Saline (PBS) and then washed twice with cold PBS. LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Invitrogen Life Technologies) was used to exclude dead cells. For intracellular staining, cells were fixed/permeabilized for 30 min with the fixation/permeabilization buffer supplied with the Foxp3/Transcription factor staining buffer set (Life Technologies eBioscience) and washed twice with permeabilization buffer. Cells were then stained intracellularly in permeabilization buffer from the Foxp3/Transcription factor staining buffer set for at least 1 h using fluorochrome-conjugated antibody against IFN-γ (XMG-1.2), IL-17A (eBio17B7), Foxp3 (FJK-16s) and/or RORγt (B2D). Each staining was performed in the presence of purified anti-mouse CD16/32 (clone 93) and 0.2 mg/ml purified rat gamma globulin (Jackson Immunoresearch). Antibodies for flow cytometry were purchased either from BD Biosciences,

BioLegend, eBioscience (Life Technologies) or R&D Systems. Flow cytometric data was acquired on an LSR II or LSR Fortessa (BD Biosciences) and analyzed using FlowJo software (TreeStar).

Scanning microscopy.

6mm ear pinnae biopsy punches were fixed in 2.5% glutaraldehyde, 1% formaldehyde, 0.12 M sodium cacodylate buffer, pH 7.4, for 90 min. Samples were postfixed with 1% OsO4 in the same buffer for 60 min and en bloc stained with 1% aqueous uranyl acetate for 1 hr. Ear punches were then dehydrated in an ethanol series and dried using a Samdri-795 critical point dryer (Tousimis Research Corp.). Punches were then positioned onto stubs with carbon adhesive tape and silver paint, coated with 5 nm gold in an EMS 575X sputter coater (Electron Microscopy Sciences), and imaged with a ZEISS Crossbeam 540 scanning electron microscope.

Confocal microscopy of ear pinnae.

Confocal microscopy was performed as previously described (2). In brief, ear pinnae were split with forceps, fixed in 1% formaldehyde solution (Electron Microscopy Sciences) overnight at 4°C and blocked in 1% BSA, 0.25% Triton X blocking buffer for 2 hours at room temperature. Tissues were then first stained overnight at 4˚C with anti-Histone H3 (citrulline R2+R8+R17) polyclonal primary antibody (Abcam ab5103) diluted in 1% BSA, 0.25% Triton X blocking buffer with rat gamma globulin (Jackson ImmunoResearch) and anti-CD16/32 (Bio-X-Cell). After washing with PBS, tissues were incubated with a goat-anti-rabbit IgG (H+L) secondary antibody conjugated to Alexa Fluor 568 (Life Technologies) for 2 hours at room temperature. Tissues were then washed, counterstained with 4', 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) and mounted in Prolong Gold. Ear pinnae images were captured on a Leica M205 stereomicroscope. Images were analyzed using Imaris Bitplane software.

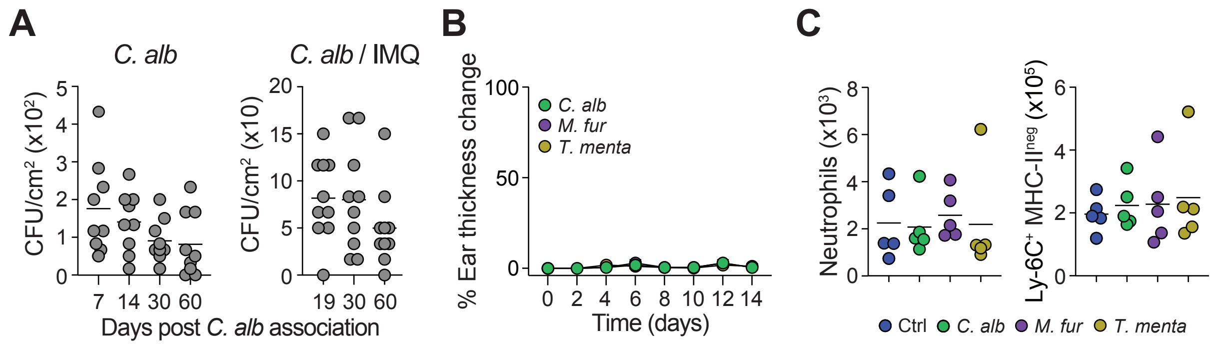
Dendritic cell and T-cell co-culture assay.

Live $CD45^{+}CD90.2^{+}CD4^{+}Foxp3^{neg}CCR6^{+}$ effector T cells (>95% purity) were sorted by fluorescence-activated cell sorting (FACS) from the cervical lymph nodes of Foxp3-GFP reporter mice one day after the last application of IMQ using a FACS Aria II cell sorter. For splenic dendritic cell (SpDC) purification, single-cell suspensions from the spleen of congenic wild-type SPF mice or *MHCII*$^{-/-}$ mice were magnetically enriched for $CD11c^{+}$ cells by positive selection using CD11c MicroBeads and MACS separation columns (Miltenyi Biotec). Purified SpDCs and $CD4^{+}$ T cells were co-cultured at a 10:1 ratio (5 x 10^{3} $CD4^{+}$ T cells) in a 96-well U-bottom plate in complete medium (RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate and nonessential amino acids, 20 mM HEPES, 100 U/ml penicillin, 100

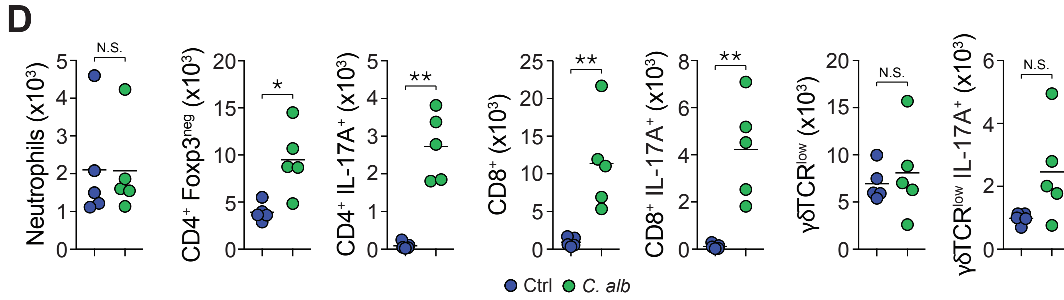
μg/ml streptomycin, 50 μM β-mercaptoethanol) for 16 h at 37°C in 5% CO_2. Brefeldin A (GolgiPlug) was added for the final 90 minutes of the culture. SpDCs were previously incubated for 2h with or without heat-killed *C. albicans* (*C. albicans*:SpDC ratio, 500:1) or *C. albicans* conditioned medium and washed before co-culture with $CD4^+$ T cells. For conditioned media, *C. albicans* was cultured aerobically in RPMI medium for 48 h at 37°C. Fungal culture was centrifugated at 3,200 x g for 20 min. Supernatant was then filtered through 0.22 μm filter (Steriflip, Millipore-Sigma, MA). Sterile filtered supernatant was used immediately or frozen at -80°C for future experiments.

References

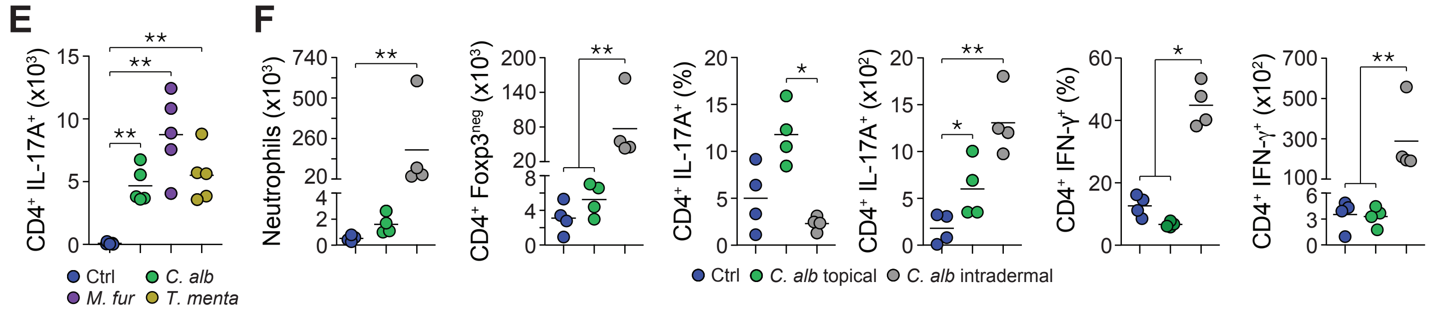
1. Naik S*, et al.* (2012) Compartmentalized control of skin immunity by resident commensals. *Science* 337(6098):1115-1119.
2. Naik S*, et al.* (2015) Commensal-dendritic-cell interaction specifies a unique protective skin immune signature. *Nature* 520(7545):104-108.



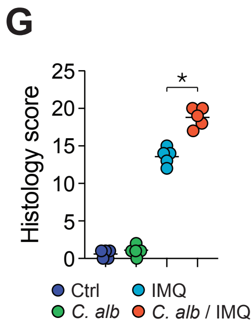
A
C. alb
C. alb / IMQ
CFU/cm² (x10²)
CFU/cm² (x10)
7 14 30 60
19 30 60
Days post C. alb association
B
% Ear thickness change
C. alb
M. fur
T. menta
Time (days)
C
Neutrophils (x10³)
Ly-6C⁺ MHC-IIneg (x10⁵)
Ctrl
C. alb
M. fur
T. menta



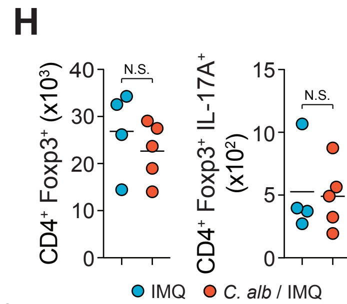
D
N.S.
Neutrophils (x10³)
CD4⁺ Foxp3neg (x10³)
CD4⁺ IL-17A⁺ (x10³)
CD8⁺ (x10³)
CD8⁺ IL-17A⁺ (x10³)
γδTCRlow (x10³)
γδTCRlow IL-17A⁺ (x10³)
Ctrl
C. alb



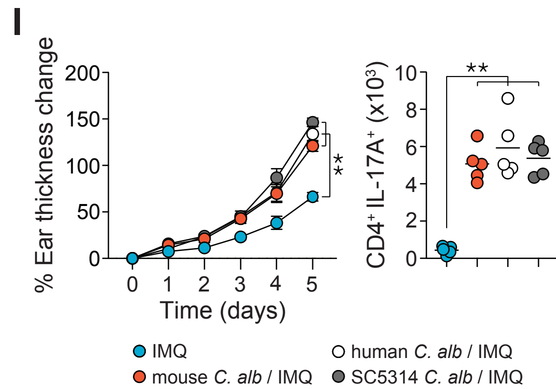
E
CD4⁺ IL-17A⁺ (x10³)
Ctrl
C. alb
M. fur
T. menta
F
Neutrophils (x10³)
CD4⁺ Foxp3neg (x10³)
CD4⁺ IL-17A⁺ (%)
CD4⁺ IL-17A⁺ (x10²)
CD4⁺ IFN-γ⁺ (%)
CD4⁺ IFN-γ⁺ (x10²)
Ctrl
C. alb topical
C. alb intradermal



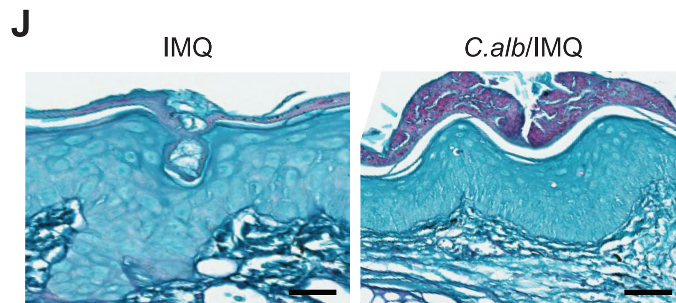
G
Histology score
Ctrl
IMQ
C. alb
C. alb / IMQ



H
N.S.
CD4⁺ Foxp3⁺ (x10³)
CD4⁺ Foxp3⁺ IL-17A⁺ (x10²)
IMQ
C. alb / IMQ



I
% Ear thickness change
Time (days)
CD4⁺ IL-17A⁺ (x10³)
IMQ
mouse C. alb / IMQ
human C. alb / IMQ
SC5314 C. alb / IMQ



J
IMQ
C.alb/IMQ

Figure S1.

(**A**) *C. albicans* colony forming unit (CFU) numbers per cm^2 on the ear pinnae 7, 14, 30 and 60 days after topical association with *C. albicans* (left) and 19, 30 and 60 days after first association with *C. albicans* in IMQ-treated SPF mice (right). (**B**) Time course of percentage of ear thickness change relative to baseline at day 0 in *C. albicans (C. alb)-, M. furfur* (*M. fur*)- and *T. mentagrophytes* (*T. menta*)-associated SPF mice. (**C**) Absolute numbers of live neutrophils (left) and Ly-6C^+MHC-II^- inflammatory monocytes (right) in the ear pinnae of unassociated control (Ctrl) mice, and *C. albicans (C. alb)*-, *M. furfur (M. fur)*- and *T. mentagrophytes* (*T. menta*)-associated SPF mice. (**D**) Absolute numbers of live neutrophils, $CD4^+Foxp3^{neg}$, IL-17A-producing $CD4^+Foxp3^{neg}$ ($CD4^+$IL-$17A^+$), $CD8^+$, IL-17A-producing $CD8^+$ ($CD8^+$IL-$17A^+$), $\gamma\delta TCR^{low}$ and IL-17A-producing $\gamma\delta TCR^{low}$ ($\gamma\delta TCR^{low}$IL-$17A^+$) cells in the ear pinnae of unassociated control (Ctrl) SPF mice and *C. albicans* (*C. alb*)-associated SPF mice. (**E**) Absolute numbers of live IL-17A-producing $CD45^+CD90.2^+TCR\beta^+CD4^+Foxp3^{neg}$ ($CD4^+$IL-$17A^+$) from the ear pinnae of control (Ctrl) SPF mice and *C. albicans (C. alb)*-, *M. furfur (M. fur)*-, and *T. mentagrophytes (T. menta)*-associated SPF mice. (**F**) Absolute numbers of live neutrophils, $CD4^+Foxp3^{neg}$, IL-17A-producing $CD4^+Foxp3^{neg}$ ($CD4^+$IL-$17A^+$), IFN-γ-producing $CD4^+Foxp3^{neg}$ ($CD4^+$IFN-γ^+), and frequencies of live IL-17A-producing $CD4^+Foxp3^{neg}$ ($CD4^+$IL-$17A^+$) and IFN-γ-producing $CD4^+Foxp3^{neg}$ ($CD4^+$IFN-γ^+) in the ear pinnae of SPF mice 7 days after intradermal injection with PBS (Ctrl), *C. albicans* topical association (*C. alb* topical) or intradermal injection with *C. albicans* (*C. alb* intradermal*)*. (**G**) Total histology score evaluated by H&E staining section of the ear pinnae of control (Ctrl), IMQ, *C. albicans (C. alb)*, and *C. albicans*/IMQ (*C. alb* / IMQ)-treated SPF mice. (**H**) Absolute numbers of live $CD4^+Foxp3^+$ and IL-17A-producing $CD4^+Foxp3^+$ in the ear pinnae of IMQ- and *C. albicans*/IMQ (*C. alb* / IMQ)-treated SPF mice. (**I**) Time course of percentage of ear thickness change relative to baseline at day 0 (left) and absolute numbers of live IL-17A-producing $CD45^+CD90.2^+TCR\beta^+CD4^+Foxp3^{neg}$ ($CD4^+$IL-$17A^+$) cells (right) in the ear pinnae from IMQ-treated SPF mice and IMQ-treated SPF mice pre-exposed to mouse *C. albicans* (*mouse C. alb* / IMQ), human *C. albicans* (*human C. alb* / IMQ) or reference strain of *C. albicans* (*SC5314 C. alb* / IMQ). (**J**) Periodic Acid-Schiff (PAS) staining of ear pinnae from IMQ and *C. albicans*/IMQ (*C. alb* / IMQ) treated SPF mice on day 5 of IMQ treatment. Scale bar = 50 μm. Cytokine production shown in this figure was analyzed following *in vitro* re-stimulation of the cells with PMA and ionomycin in the presence of brefeldin A. Results are representative of 2-3 independent experiments. *p-value < 0.05, **p-value < 0.01; N.S., not significant.

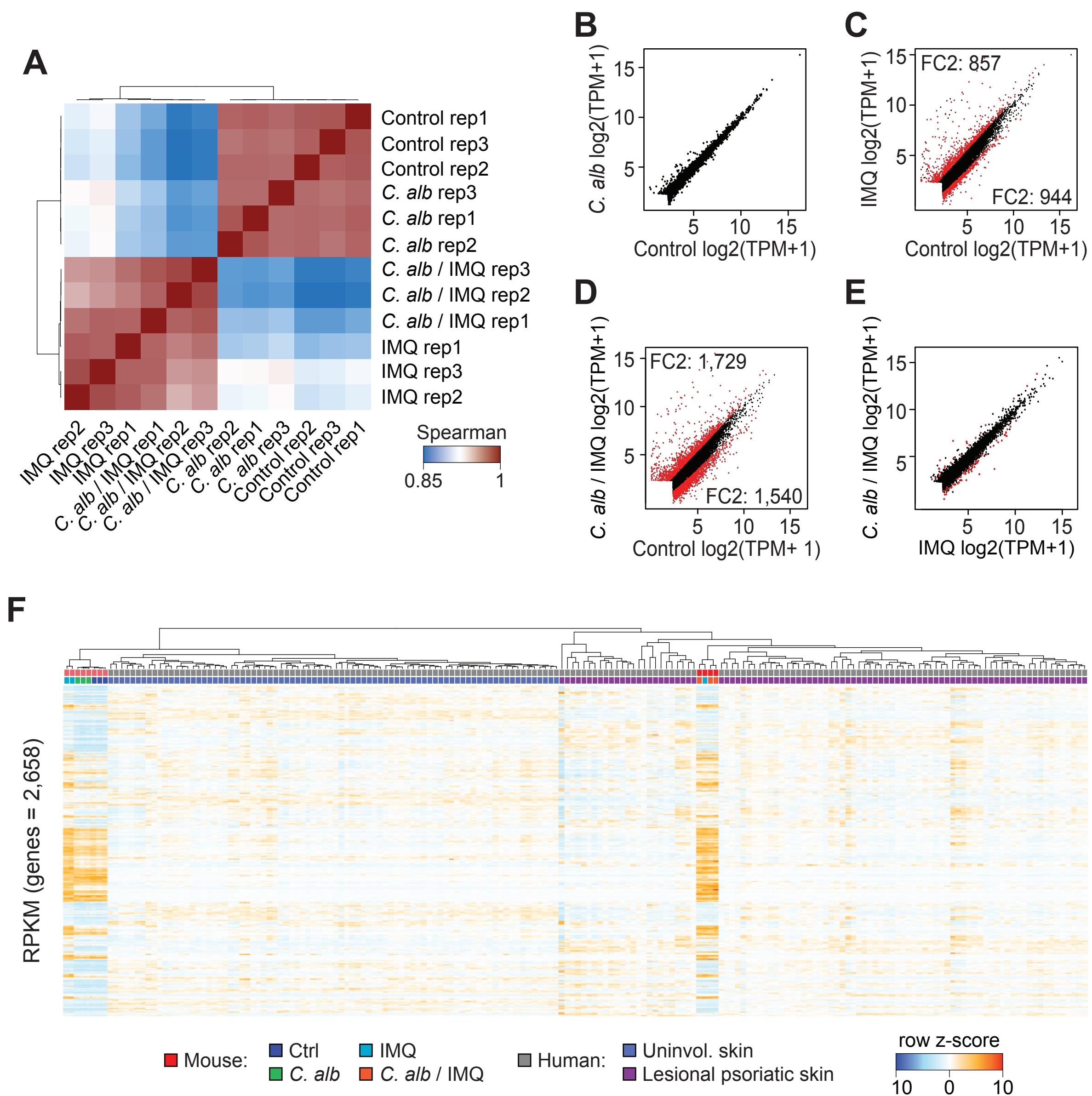


Figure S2.
(**A**) Spearman correlation co-efficient heat map for whole tissue RNA-seq samples from ear pinnae expressed with transcripts per million greater 4. There are two distinct clusters separated by treatment with IMQ. (**B-E**) Scatter plots for whole tissue RNA-seq between naïve (Control) and *C. albicans (C. alb)*-treated samples (**B**), naïve (Control) and IMQ-treated samples (**C**), naïve (Control) and *C. albicans*/IMQ (*C. alb* / IMQ)-treated samples (**D**) and IMQ and *C. albicans*/IMQ (*C. alb* / IMQ)-treated samples (**E**). Red dots show 2-fold significantly differently expressed genes (FDR < 5%). Data is normalized to transcripts per million (TPM) and log2 transformed. (**F**) Heat map showing clustering of gene expression (normalized to reads per kilobase per million (RPKM)) of human normal (uninvolved) skin (blue), human lesional psoriatic skin (purple) and murine control samples (dark blue), murine *C. albicans (C. alb)*-treated samples (green), IMQ-treated samples (light blue) and *C. albicans*/IMQ (*C. alb* / IMQ)-treated samples (orange). Values are plotted as row z-score.

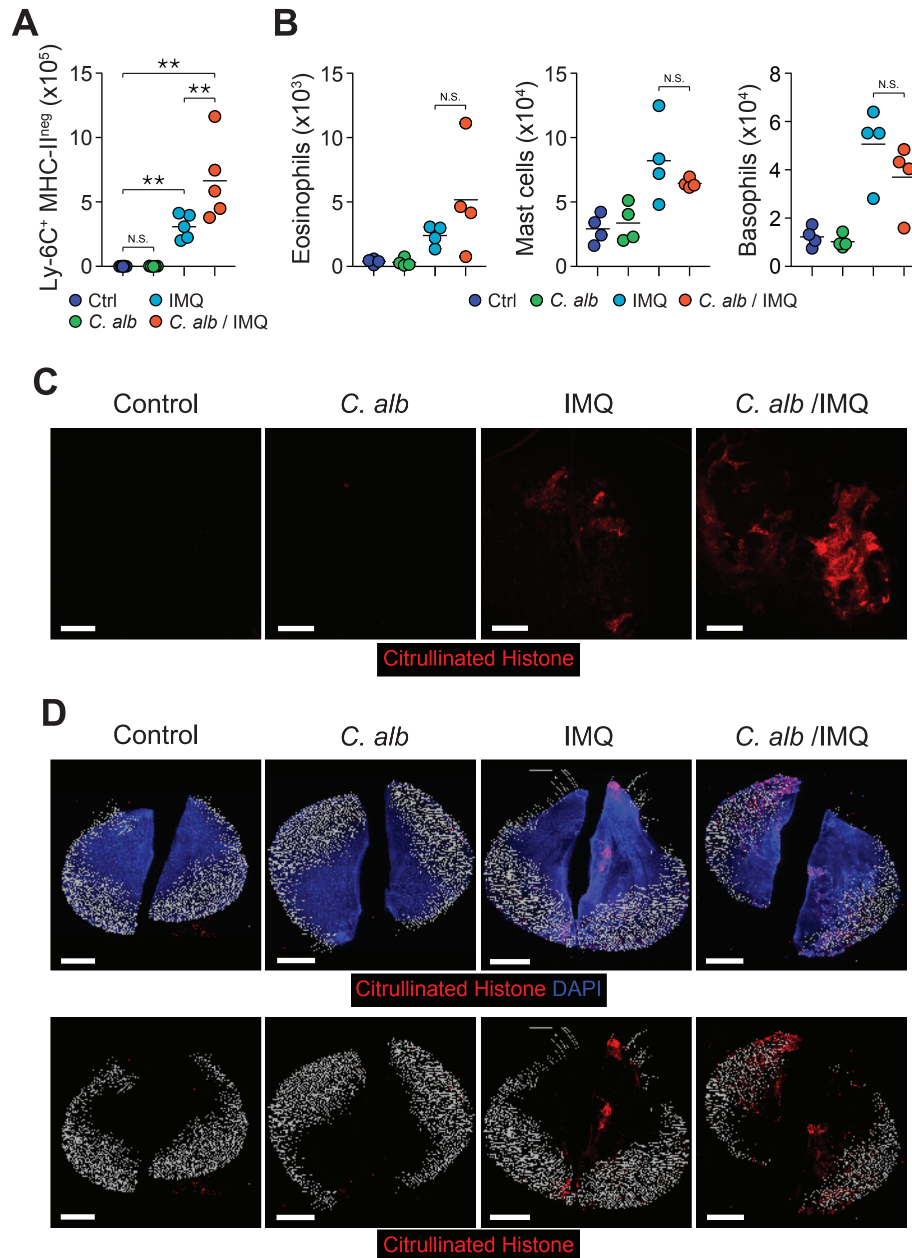
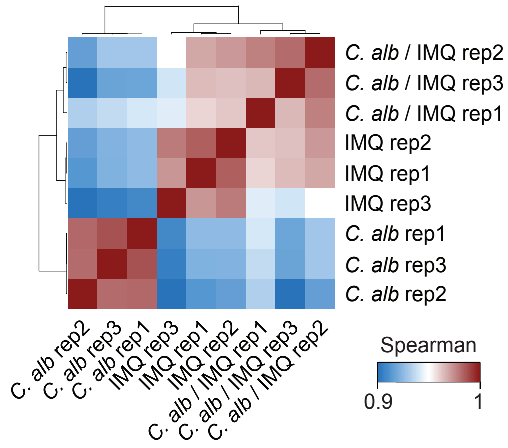


Figure S3.

(**A**) Absolute numbers of live Ly6C^{+}MHC-II^{-} inflammatory monocytes in the ear pinnae of control (Ctrl) mice, *C. albicans (C. alb)*-, IMQ-, and *C. albicans*/IMQ (*C. alb* / IMQ)-SPF treated mice. (**B**) Absolute numbers of live eosinophils, mast cells and basophils in the ear pinnae of control (Ctrl) SPF mice, *C. albicans (C. alb)*-, IMQ-, and *C. albicans*/IMQ (*C. alb* / IMQ)-treated SPF mice. (**C**) Representative imaging of high magnification of whole mouse ear pinnae section from control SPF mice and *C. albicans (C. alb)*-, IMQ-, and *C. albicans*/IMQ (*C. alb* / IMQ)-treated SPF mice showing citrullinated histone H3^{+} cells (red). Scale bars = 50 μm. (**D**) Representative imaging of whole ear pinnae from control SPF mice and *C. albicans (C. alb)*-, IMQ-, and *C. albicans*/IMQ (*C. alb* / IMQ)-treated SPF mice, showing NETs, as demonstrated by positive citrullinated histone H3+ staining (red) and DAPI (blue). Scale bars = 3 mm. Results are representative of 2-3 independent experiments. *p-value < 0.05, **p-value < 0.01; N.S., not significant.

A



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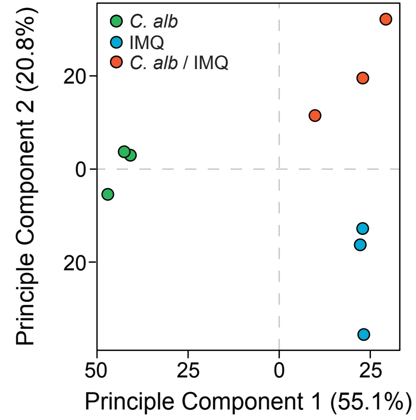

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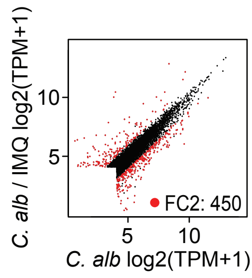

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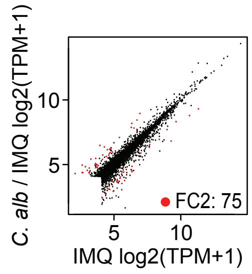

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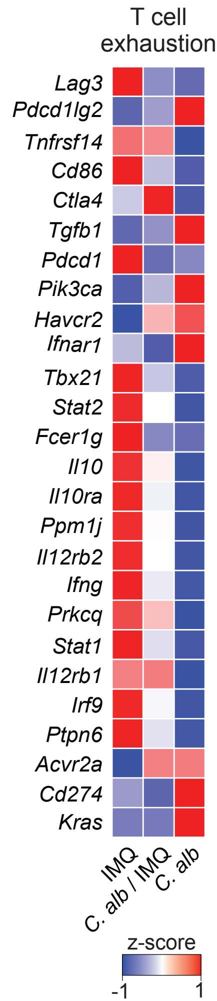

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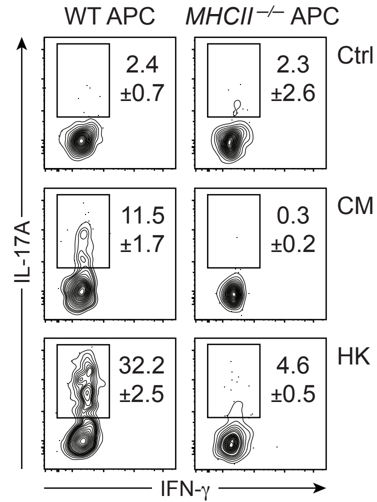

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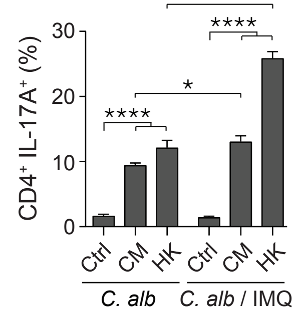

Figure S4.

(**A**) Spearman correlation co-efficient heat map for TCRβ$^+$CD90.2$^+$CD4$^+$Foxp3^{neg} T cell RNA-seq samples from the ear pinnae of IMQ-, *C. albicans* (*C. alb*)- and C. *albicans*/IMQ (*C. alb* / IMQ)-treated SPF mice in replicates. (**B**) Principle component analysis (PCA) for RNA-seq samples from TCRβ$^+$CD90.2$^+$CD4$^+$Foxp3^{neg} T cells from the ear pinnae of *C. albicans* (*C. alb*)-, IMQ- and *C. albicans*/IMQ (*C. alb* / IMQ)-treated SPF mice. (**C-D**) Scatter plot showing log normalized transcripts per million (TPM) for live TCRβ$^+$CD90.2$^+$CD4$^+$Foxp3^{neg} T cells from ear pinnae comparing *C. albicans* (*C. alb*)- versus *C. albicans*/IMQ (*C. alb* / IMQ)-treated samples (**C**) and IMQ- versus *C. albicans*/IMQ (*C. alb* / IMQ)-treated samples (**D**). Red dots show genes significantly different regulated (fold change > 2, FDR < 5%). (**E**) Heat map showing row z-score of genes associated with T cell exhaustion pathway from IPA analysis (Fig. 3F). (**F-G**) CD4$^+$Foxp3^{neg}CCR6$^+$ T cells from the skin draining LNs of *C. albicans*-treated and/or *C. albicans*/IMQ-treated SPF mice were co-cultured with *Rag1*$^{-/-}$ (WT) or *MHCII* $^{-/-}$ splenic dendritic cells untreated (Ctrl) or pre-incubated with *C. albicans* conditioned media (CM) or heat killed *C. albicans* (HK). Flow plots in panel F and bar graph in panel G illustrate the frequencies of CD4$^+$ T cells expressing IL-17A in overnight co-cultures. Cytokine production was analyzed following *in vitro* re-stimulation of the cells with PMA and ionomycin in the presence of brefeldin A. For panels F and G, results are representative of 3 independent experiments. *p-value < 0.05, ****p-value < 0.0001.

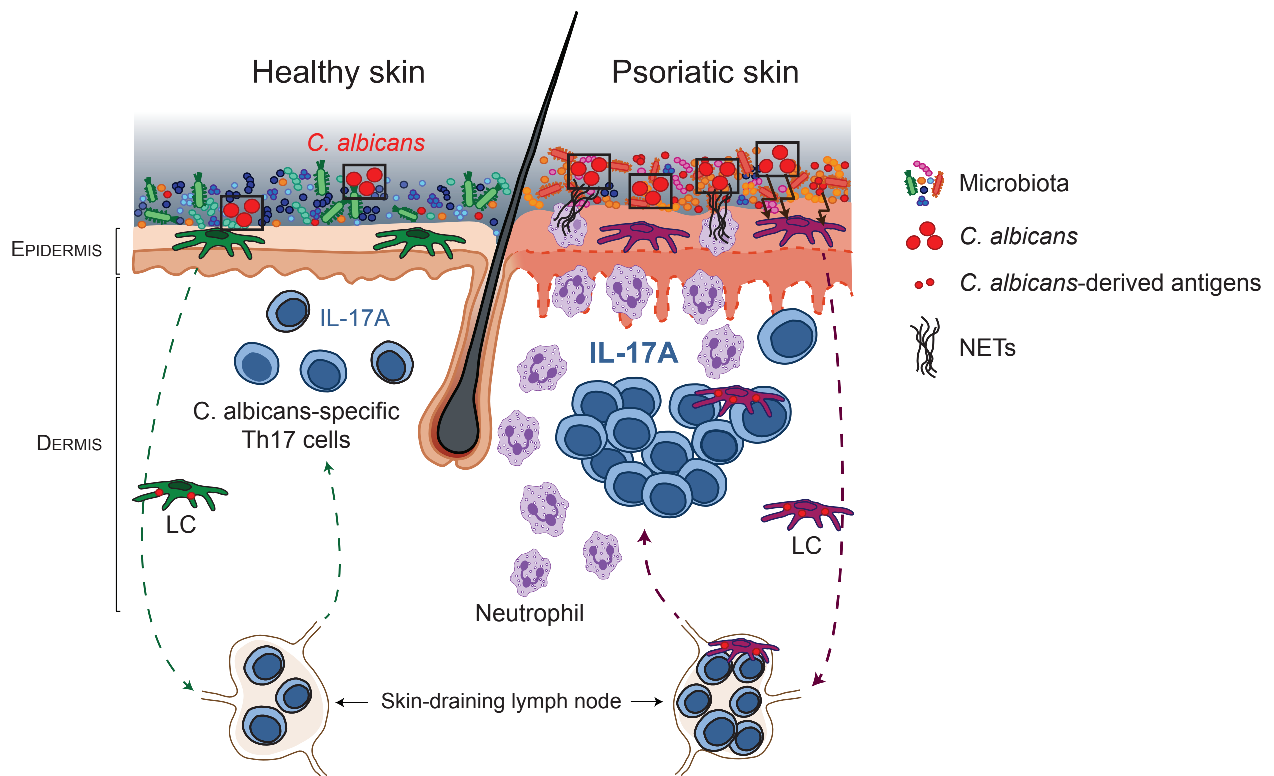

Figure S5. Adaptive immunity to skin fungi directly promotes skin inflammation.
Our work supports the following model: Upon exposure of the skin to *C. albicans*, Langerhans cells (LCs) capture *C. albicans*-derived antigens and migrate to the skin-draining lymph nodes where they induce *C. albicans*-specific IL-17A-producing $CD4^{+}$ T cells (Th17). In healthy skin (left side), those Th17 cells travel back to the skin without causing inflammation. In the context of inflammation, such as after treatment with IMQ (psoriatic skin, on the right side), *Candida*-specific Th17 cells expand in response to *C. albicans*-derived antigen presented by activated DCs (possibly LCs); of note additional priming of *C. albicans* specific T cells may also occur. These expanded Th17 cells exacerbate the recruitment of neutrophils and the formation of neutrophils extracellular traps (NETs).