**Fungal Infection**

**Oral epithelial cells orchestrate innate type 17 responses to Candida albicans through the virulence factor candidalysin**


Candida albicans is a dimorphic commensal fungus that causes severe oral infections in immunodeficient patients. Invasion of *C. albicans* hyphae into oral epithelium is an essential virulence trait. Interleukin-17 (IL-17) signaling is required for both innate and adaptive immunity to *C. albicans*. During the innate response, IL-17 is produced by γδ T cells and a poorly understood population of innate-acting CD4⁺ αβ T cell receptor (TCRαβ)⁺ cells, but only the TCRαβ⁺ cells expand during acute infection. Confirming the innate nature of these cells, the TCR was not detectably activated during the primary response, as evidenced by Nur77eGFP mice that report antigen-specific signaling through the TCR. Rather, the expansion of innate TCRαβ⁺ cells was driven by both intrinsic and extrinsic IL-1R signaling. Unexpectedly, there was no requirement for CCR6/CCL20-dependent recruitment or prototypical fungal pattern recognition receptors. However, *C. albicans* mutants that cannot switch from yeast to hyphae showed impaired TCRαβ⁺ cell proliferation and IL17α expression. This prompted us to assess the role of candidalysin, a hyphal-associated peptide that damages oral epithelial cells and triggers production of inflammatory cytokines including IL-1. Candidalysin-deficient strains failed to up-regulate IL17α or drive the proliferation of innate TCRαβ⁺ cells. Moreover, candidalysin signaled synergistically with IL-17, which further augmented the expression of IL-1α/β and other cytokines. Thus, IL-17 and *C. albicans*, via secreted candidalysin, amplify inflammation in a self-reinforcing feed-forward loop. These findings challenge the paradigm that hyphal formation per se is required for the oral innate response and demonstrate that establishment of IL-1– and IL-17–dependent innate immunity is induced by tissue-damaging hyphae.

**INTRODUCTION**

The commensal fungus *Candida albicans* colonizes human mucosal surfaces. Changes in immune competency or oral mucosal barriers promote development of oropharyngeal candidiasis (OPC or thrush), an opportunistic infection prevalent in HIV and AIDS, iatrogenic immunosuppression, head-neck irradiation, Sjögren’s syndrome, and infancy (1, 2). Patients with mutations in genes that affect T helper 17 (TH17 or T(H)17) cells or the interleukin-17 receptor (IL-17R) signaling pathway are extremely susceptible to chronic mucocutaneous candidiasis (3). Neutralizing antibodies that occur in AIRE deficiency or as a result of biologic therapy for autoimmunity can also cause mucosal candidiasis (4). Mice with IL-17R signaling deficits are similarly susceptible to *C. albicans* infections (5, 6). Unlike humans, *C. albicans* is not a commensal microbe in rodents, and therefore, mice are immunologically naïve to this fungus (7, 8). Nonetheless, during recall infections with *C. albicans*, mice mount vigorous TH17 responses that augment innate immunity, in keeping with humans where the memory response to *C. albicans* is TH17-dominated. During the naïve response, IL-17 is produced by several innate lymphocyte subsets, but the only cells that expand robustly upon infection belong to an oral-resident innate TCRαβ⁺ population, sometimes called “natural” TH17 cells (9).

An essential virulence trait of *C. albicans* is its ability to transition from its commensal yeast form to an invasive and cell-damaging hyphal state. In the adaptive immune response, dendin-1 expressed on myeloid cells recognizes β-glucan components of the fungal cell wall that are exposed during the hyphal transition. This leads to the production of IL-6 and IL-23, which promote TH17 cell differentiation (10–12). Unexpectedly, however, neither CARD9 nor IL-6 is required for the innate IL-17 response to OPC (9, 13). Therefore, it has been unclear how innate IL-17–expressing cells are activated during primary *C. albicans* infections and why this only occurs in response to invasive, tissue-damaging hyphae.

The initiating event in OPC is the exposure of oral epithelial cells (OECs) to *C. albicans*. Hyphae, but not yeast, cause lysis and danger responses in OECs, including production of cytokines and chemokines [IL-6, IL-1α/β, GM-CSF (granulocyte-macrophage colony-stimulating factor), G-CSF (granulocyte colony-stimulating factor), and CCL20], antimicrobial peptides (β-defensins), and damage-associated molecular patterns (IL-1r and S100A8/9) (14, 15). This OEC activation program is triggered by candidalysin, an amphipathic pore-forming peptide derived from the hyphal-specific ECE1 (extent of cell elongation 1) gene product (16). Many of the cytokines induced by candidalysin are associated with TH17 responses or recruitment (for example, IL-1α/β, IL-6, and CCL20), which led us to postulate that candidalysin might influence the generation of the early IL-17 response to infection.

Here, we demonstrate that innate oral TCRαβ⁺ cells express IL-17 and proliferate in response to *C. albicans* infection without discernible...
activation of the TCR or a requirement from canonical fungal pattern recognition receptors. Instead, proliferation of innate IL-17^CCR6^ cells and expression of IL-17 were regulated by candidalysin-driven IL-1α/β. Consistently, Il1r1^−/−^ mice are susceptible to OPC, with redundant activities in hematopoietic and nonhematopoietic compartments. Moreover, candidalysin and IL-17 signal synergistically in OECs to augment the expression of antifungal response genes. Therefore, innate IL-17–induced responses are triggered specifically in response to candidalysin secreted from hyphae, revealing unexpected differences in how activation of innate versus adaptive IL-17–dependent immunity is controlled.

**RESULTS**

*C. albicans* induces proliferative expansion of innate oral IL-17^CCR6^ cells

We previously showed that IL-17 is induced in the oral mucosa within 24 hours of infection with *C. albicans*. Expression of IL-17 diminishes concomitantly with clearance, typically in 3 days (5, 8). Using Il17a^eGFP^ fate-tracking mice (17), we found that IL-17 produced during acute oral *C. albicans* challenge originates dominantly from tongue-resident γδ T cells and an unconventional population of innate-like CD4^+^ TCRβ^+^ cells (9). IL-17 production by group 3 innate lymphoid cells (ILC3s) has been reported in OPC (18), although their frequency is below the limit of detection in our hands. These IL-17^CCR6^ cells are sometimes termed “natural” Tγ17 cells (9, 19, 20), but here, we refer to them as "innate TCRβ^+^ cells," as per Kashem et al. (21). In the oral cavity, the innate IL-17^CCR6^ cells reproducibly expand about twofold after encounter with *C. albicans*, whereas the frequency of IL-17^γδ^ T cells is low and does not change during infection (Fig. 1A) (9). *C. albicans*–dependent expansion of oral TCRβ^+^ cells was similarly observed in non–fate-tracking mice, starting at day 1 after infection and peaking at day 2 after infection (Fig. 1B and C, and fig. S1).

The expansion of innate TCRβ^+^ cells could be due to proliferation, survival, recruitment, or a combination. To assess proliferation, we infected wild-type (WT) mice orally and measured intracellular Ki67 by flow cytometry. On day 1, Ki67^CCR6^ T cells were more frequent in the infected oral mucosa compared to sham controls (Fig. 1B). More profound proliferation was observed at day 2, where we consistently saw a twofold increase in the percent and total cell number in *C. albicans*–infected mice compared to sham-infected controls harvested within the same experiment (Fig. 1C). Proliferation was confirmed by intracellular staining for proliferating cell nuclear antigen (PCNA) (Fig. 1D). The expansion of TCRβ^+^ cells by *C. albicans* was similar in mice from different vendors (fig. S2A), and the proliferating cells exhibited a diverse TCRβ repertoire (fig. S2B). *C. albicans*–induced proliferation of TCRβ^+^ cells was limited to the local site of infection (tongue) because there was no change in the baseline frequency of replicating CD4^+^ TCRβ^+^ cells in the draining cervical lymph node (cLN) (Fig. 1E). These data confirm our previous findings that IL-17 is expressed in local oral tissue but not in cLN during a primary *C. albicans* infection (8). Thus, the twofold expansion of TCRβ^+^ cells during OPC can be accounted for by local proliferation at the site of infection.

Oral-resident TCRβ^+^ cells express CCR6, a marker of IL-17^+^ cells and a receptor for the chemokine CCL20 (9). To determine whether signaling through the CCL20/CCR6 axis was required for immunity to OPC, we analyzed responses in Ccr6^−/−^ mice and mice given neutralizing anti-CCL20 antibodies (22). Innate TCRβ^+^ cells in Ccr6^−/−^ mice showed a similar proliferation capacity to WT controls after *C. albicans* infection (Fig. 2A and fig. S1). There was also no difference in the baseline population of TCRβ^+^ cells in Ccr6^−/−^ mice compared to WT mice (fig. S3). Resistance to OPC was similar in Ccr6^−/−^ and WT mice, with low oral fungal burdens at 4 days after infection (Fig. 2B). Similar results were obtained when mice were administered anti-CCL20 antibodies (Fig. 2C). Accordingly, the baseline frequency and the *C. albicans*–induced expansion of TCRβ^+^ cells in the oral mucosa are independent of CCL20 and CCR6, although we cannot rule out the involvement of other chemotactic factors.

**Oral-resident innate TCRβ^+^ cells drive anti-*Candida* immunity independently of TCR signaling or specificity**

Mice are naïve to *C. albicans*, and animals lacking lymphocytes (for example, Rag1^−/−^ and Il7ra^−/−^ mice) are highly susceptible to OPC (8, 9). To determine whether *C. albicans*–induced expansion of innate TCRβ^+^ cells requires antigen-specific signaling, we used Nur77^GFP^ reporter mice, which report the kinetics and magnitude of TCR signaling through the expression of green fluorescent protein (GFP) driven by the promoter of the immediate-early gene Nrl4al (Nur77) (23). First, to verify that TCR activation could be visualized in oral T cells, we administered agonistic anti-CD3 antibodies to WT mice to activate the TCR nonspecifically; this treatment effectively induced GFP fluorescence in TCRβ^+^ cells from the tongue (Fig. 3A). Next, to determine whether TCR signaling was activated during the innate response, we challenged Nur77^GFP^ mice orally with *C. albicans* or PBS (sham), and GFP fluorescence in oral TCRβ^+^ cells was assessed at days 1 and 2 after infection. As expected, T cells from sham-infected mice showed a low but detectable baseline level of tonic GFP expression (23). In mice infected with *C. albicans* for 1 to 2 days (1^st^ infection), there was the same baseline GFP fluorescence, as seen in sham cohorts, indicating that there was no TCR signaling upon the first encounter with *C. albicans* and confirming the innate nature of these cells (Fig. 3A).

The Nur77^GFP^ reporter system can also be used to compare TCR signaling strength, so we assessed the frequency of GFP^hi^ cells (that is, T cells with more potent TCR signaling) in mice given a primary (1^st^) or a secondary (2^nd^) *C. albicans* infection. Again, there were no differences between sham–treated mice and those receiving a 2-day (1^st^) challenge (Fig. 3B). To verify that *C. albicans*–specific signaling through the TCR could be observed, if present, we generated a 2^nd^ response by subjecting mice to infection and then rechallenging them after 6 weeks; this regimen induces an Ag-specific Tγ17 response that enhances fungal clearance (8). There was an increased frequency of GFP^hi^ TCRβ^+^ cells in the tongues from rechallenged mice, demonstrating that Ag-specific responses can be visualized with Nur77^GFP^ mice in the context of a recall response (Fig. 3B). Therefore, consistent with the naïve state of mice with respect to *C. albicans*, TCR signaling appears not to be activated during the expansion of TCRβ^+^ cells in an innate infection.

**Pattern recognition receptors required for the adaptive response to *C. albicans* are dispensable for the activation of innate TCRβ^+^ cells**

Dectin-1 (*Clec7a*) is a C-type lectin receptor (CLR) used by phagocytes to sense β-glucan carbohydrates that are exposed on *C. albicans* during filamentation. Dectin-1 induces IL-23 and IL-6 in antigen-presenting cells (APCs), skewing to a Tγ17 phenotype (11, 24). However, it was not known whether dectin-1 signals similarly drive IL-17 production during the innate response. In Clec7a^−/−^ mice, there was a rapid and
The secreted peptide candidalysin activates innate TCRαβ⁺ cell expansion

Hyphal formation is a key virulence trait for C. albicans. Consistently, a C. albicans mutant “locked” in the yeast phase [efg1Δ/Δ (29)] did not induce Il17a or expression of IL-17-dependent genes, such as Defb3 (β-defensin 3), Il1b, or Cxcl20 (fig. S4A). In its hyphal state, C. albicans secretes candidalysin, a short, amphipathic pore-forming peptide. Candidalysin, encoded by the ece1 gene, destabilizes epithelial membranes and triggers OEC production of cytokines such as IL-1α, IL-1β, and IL-6 (16). Because these cytokines are linked to T H 17 responses (30), we hypothesized that candidalysin might serve as an activator of innate TCRαβ⁺ cell expansion and IL-17 production. Il17aΔ/YFP reporter mice were infected with C. albicans strains lacking ECE1 (ece1Δ/Δ) or an ECE1-revertant control (“Rev”). Mice infected with ece1Δ/Δ exhibited reduced expansion of IL-17⁺ TCRαβ⁺ cells at day 2 after infection. In contrast, mice challenged with the ECE1-Rev strain showed robust TCRαβ⁺ expansion (Fig. 5A). Similar results were obtained in WT mice (Fig. 5, B and C). The diminished TCRαβ⁺ expansion in ece1Δ/Δ-infected mice correlated with reduced proliferation (Fig. 5B, bottom), observed at both days 2 and 3 after infection (fig. S4B). By day 5, the infection was resolved, and the T cell proliferative response had returned to baseline. At day 2, when cells were harvested, fungal loads were comparable, indicating that the impaired TCRαβ⁺ cell proliferation was not due to reduced exposure to fungal antigens (Fig. 5D). Therefore, candidalysin is required for the expansion of innate TCRαβ⁺ cells during acute oral C. albicans infection.

Consistent with the reduced TCRαβ⁺ cell proliferation, mice infected with strains lacking ECE1 or just the candidalysin sequence (ClysΔ/Δ) showed impaired induction of Il17a mRNA expression (Fig. 5E), as well as Defb3 and S100a9 (Fig. 5F). Neutrophil mobilization to the tongue, which is regulated, in part, by IL-17 signaling (5, 31, 32), was also reduced in ece1Δ/Δ-challenged mice (Fig. 5G). We verified that the activation of TCRαβ⁺ proliferation is induced in response to an unrelated C. albicans strain, HUN96 (33), a clinical isolate that expresses ECE1, induces c-Fos, and damages OECs in vitro (fig. S4C). C. albicans secretes multiple virulence factors, particularly secreted aspartyl proteinases (SAPs). To determine whether the innate IL-17 response was specific to candidalysin, we evaluated TCRαβ⁺ proliferation after infection with fungal strains lacking the hypha-associated SAP genes (SAP4-6) (34, 35). Notably, there was no defect in TCRαβ⁺ proliferation in

robust proliferation of innate TCRαβ⁺ cells after oral C. albicans infection, indicating that dectin-1 is not required for innate TCRαβ⁺ cell expansion (Fig. 4A and fig. S1). A similar proliferative response occurred in mice lacking CARD9, a key adaptor downstream of dectin-1 and other CLRs (Fig. 4B and fig. S1) (25–27). Toll-like receptor 2 (TLR2) has also been implicated in recognition of C. albicans through the engagement of hyphae (24, 28). However, there was a robust proliferation of innate TCRαβ⁺ cells in Tlr2⁻/⁻ mice upon 1° C. albicans challenge (Fig. 4C and fig. S1).

To determine whether TLR2, dectin-1, or CARD9 was necessary for clearing C. albicans in acute oral infection, we assessed fungal loads 5 days after infection. Clearance was not impaired in mice lacking dectin-1 (Fig. 4, D and E), consistent with our previous report that CARD9 is dispensable for innate immunity to OPC (13). Similarly, resolution of C. albicans was not impaired in Tlr2⁻/⁻ mice (Fig. 4, D and E). Hence, TLR2 or dectin-1/CARD9 signaling is dispensable for the expansion of innate TCRαβ⁺ cells during innate immunity to OPC.
response to infection with sap4-6Δ/Δ strain compared to the parent strain (fig. S4D).

**Innate TCRαβ+ cell proliferation in the oral mucosa is dependent on IL-1α/β signaling**

Candidalysin elicits production of several cytokines known to affect the differentiation or proliferation of some IL-17–producing cells, such as IL-6, IL-1α, and IL-1β (16). In the tongues of mice subjected to 1° Candida albicans infection, expression of Il1b mRNA was induced in an ECE1-dependent manner (Fig. 6A). Expression of Il1a showed a similar trend, but Il6 was not induced in this time frame (Fig. 6A). Il6−/− mice are resistant to acute OPC (9), and here, we verified that the proliferation of innate TCRαβ+ cells occurred normally in the absence of IL-6 (fig. S5A). In contrast, there was no expansion or proliferation of oral innate TCRαβ+ cells in infected Il1r1−/− mice (fig. 6B and fig. S1). Consistently, Il1r1−/− mice were more susceptible to OPC than WT, although fungal burdens were not as high as in mice with an IL-17R signaling defect [here, Act1 deficiency (36)] (Fig. 6C). We next used neutralizing antibodies against either IL-1α or IL-1β (or both) to delineate the specific IL-1 family member needed to drive proliferation. As shown, blockade of either IL-1α or IL-1β impaired TCRβ+ cell proliferation, with a somewhat stronger effect under IL-1β–blocking conditions (Fig. 6D).
IL-1 signaling can occur on most cell types, including both hematopoietic and nonhematopoietic compartments. To identify the key cell type(s) that responded to IL-1, we irradiated congenically marked WT and Il1r1−/− mice and reconstituted them with the same or reciprocal bone marrow (BM). After 6 weeks, mice were infected orally with C. albicans, and proliferation of donor TCRβ+ cells was assessed. As expected, Il1r1−/− mice given Il1r1−/− BM showed impaired proliferation compared to WT counterparts (Fig. 6E). Unexpectedly, however, regardless of the source of BM, C. albicans infection induced TCRαβ+ cell proliferation under both experimental chimera conditions (that is, WT → Il1r1−/− and Il1r1−/− → WT). There was some variation in the percentage of Ki67+ cells at baseline (sham) among cohorts, but in all cases, there was an increase in proliferation after C. albicans infection. This result suggests that there are redundant IL-1R–dependent signals in radiosensitive and radioresistant compartments with respect to driving innate TCRαβ+ cell proliferation. To verify this unexpected finding, we created mixed chimeras, in which irradiated WT mice were reconstituted with a 50:50 mix of Il1r1−/− and WT BM. Again, both WT and IL-1R–deficient cells proliferated robustly in response to infection (fig. S5B). As a third approach, we performed adoptive transfer experiments using BM from mice lacking Il1r1, specifically in TCRαβ+ cells (37). Again, TCRαβ+ cells proliferated after OPC (fig. S5C), indicating that IL-1 signals occur in both hematopoietic and nonhematopoietic cells. Collectively, these data suggest the existence of IL-1 responder cells in both compartments that indirectly drive TCRαβ+ cell proliferation. We also noted that the baseline Ki67 staining in innate TCRαβ+ cells was reduced in Il1r1−/− cells compared to WT, which was most apparent in the mixed BM chimera. These results suggested that IL-1R–driven signals may directly support T cell proliferation under homeostasis. Nonetheless, only when there is a global deficiency in the IL-1R is there a failure of TCRαβ+ cells to proliferate during C. albicans infection.

**Candidalysin and IL-17 synergistically signal and amplify antifungal immunity in OECs**

Candidalysin signaling in OECs up-regulates inflammatory cytokines, such as IL-6, IL-1β, G-CSF, and CCL20. Many of these genes are also targets of IL-17 in OECs (32). IL-17 is generally a modest activator of signaling and gene expression compared to other inflammatory stimuli and, instead, mediates its activities by signaling synergistically with other cytokines. For example, Candidalysin and IL-17 synergistically signal and amplify antifungal immunity in OECs (33).
or additively with cytokines, such as tumor necrosis factor–α (TNFα). Accordingly, we hypothesized that IL-17 and candidalysin might signal cooperatively in OECs to drive antifungal immune responses. To test this idea, we infected human buccal epithelial cells (TR146 cell line) in vitro with *C. albicans* (WT parent strain, ece1Δ/Δ or Rev) in the presence or absence of IL-17. After 24 hours, conditioned supernatants were assessed for candidalysin-inducible cytokines and chemokines by Luminex. There was an additive or synergistic effect of IL-17 with candidalysin in up-regulating cytokines and chemokines, including IL-1β, IL-6, CCL20, and G-CSF (Fig. 7A). To determine whether this synergy was mediated directly by candidalysin, we treated cells with sublytic concentrations of candidalysin (15 μM) together with IL-17 (200 ng/ml) for 24 hours. There was a synergistic or additive induction of cytokines in the presence of IL-17 (Fig. 7B). TNFα showed similar cooperation with candidalysin (fig. S6). However, IL-22, which is also produced by type 17 cells and is induced in the tongue during OPC (5), did not synergize with candidalysin (fig. S6). Thus, IL-17, TNFα, and candidalysin cooperatively enhance inflammatory signaling in OECs.

Another function of candidalysin is to induce cell damage, presumed to facilitate fungal access to nutrients and invasion into deep tissue. **Fig. 5. Candidalysin drives the proliferation of innate IL-17–producing TCRαβ+ cells. (A) Il17aeYFP mice were infected with *C. albicans* (ece1Δ/Δ or Rev), and homogenates were prepared 2 days after infection. Staining of CD45 and yellow fluorescent protein (YFP) in lymphocyte gate is shown. Data are representative of two experiments. (B) WT mice were infected with the indicated strains of *C. albicans*, and expansion (top) and proliferation (bottom) of oral TCRβ+ cells were analyzed at day 2 after infection. Data are representative of three experiments. (C) Fold expansion of TCRβ+ cells after infection with ece1Δ/Δ or Rev strains. Data are pooled from four experiments. (D) Fungal loads were assessed at day 2 after infection. Bar represents the geometric mean. Data were pooled from two experiments. (E and F) Tongue homogenates were prepared 2 days after infection with the indicated *C. albicans* strains. Total mRNA was subjected to quantitative real-time polymerase chain reaction (qPCR) normalized to *Gapdh*. Graphs show mean + SEM normalized to sham. Data are compiled from seven to eight mice per group from two independent experiments. (G) Percentage of CD11b+Ly6Ghi cells in the tongue was assessed at day 2 after infection. Graphs indicate mean + SEM, as compiled from three experiments. Statistics were analyzed by Student’s t test or ANOVA. PMNs, polymorphonuclear leukocytes. *P < 0.05, **P < 0.01, and ***P < 0.001.
Fig. 6. IL-1 activates innate TCRαβ+ cell proliferation and antifungal immunity in a T cell–intrinsic and –extrinsic manner. (A) WT mice were infected with the indicated C. albicans strains, and gene expression was measured on day 2 after infection. Data are means ± SEM normalized to sham, from seven to eight mice per group in two experiments. (B) Expansion and proliferation of TCRαβ+ cells in Il1r1−/− mice at day 2 after infection. Data are from three experiments. (C) Fungal burdens in the indicated mice were quantified from two experiments on day 5 after infection. (D) WT mice were administered anti–IL-1α, anti–IL-1β, or isotype control antibodies (1.0 mg per mouse used alone or 0.5 mg each when used together) on day −1 relative to infection. Proliferation of oral TCRαβ+ cells was assessed at day 2 after infection. Data are representative of two experiments. (E) Reciprocal adoptive transfers of femoral BM were performed in WT or Il1r1−/− mice, and proliferation of oral TCRαβ+ cells was determined. Experimental chimera results are representative of two experiments; control chimera data are from one experiment. Data were analyzed by Student's t test or ANOVA. *P < 0.05, **P < 0.01, and ***P < 0.001.
Candidalysin and IL-17 signal synergistically or additively in OECs. (A) TR146 OECs were untreated (U; gray bars) or stimulated with IL-17 (200 ng/ml; black bars). Cells were infected with WT C. albicans (Bwp17 + Clp30; “Parent”), ece1Δ/Δ, or the Rev for 24 hours. Supernatants were analyzed by Luminex (IL-1β, IL-6, and G-CSF) or enzyme-linked immunosorbent assay (ELISA) (CCL20). Graphs indicate mean ± SEM. Data are representative of two experiments. (B) TR146 cells were untreated (U; gray bars) or stimulated with IL-17 (200 ng/ml), or candidalysin (15 μM) for 24 hours and analyzed as in (A). (C) TR146 cells were incubated with C. albicans ± IL-17 (200 ng/ml). LDH in supernatants was evaluated after 24 hours, representative of three experiments. (D) TR146 cells were treated with TNFα (20 ng/ml), IL-1β (200 ng/ml), or candidalysin (15 μM) for 5 min. Lysates were immunoblotted for phospho-IκBα and total IκBα. (E) TR146 cells were incubated with TNFα (20 ng/ml), IL-1β (200 ng/ml), or candidalysin (15 μM) for 30 min or 2 hours. Lysates were immunoblotted for c-Fos, phospho-MKP1, or Actin. Data are representative of two independent experiments. (F) TR146 cells were transfected with c-Fos small interfering RNA (siRNA) and stimulated for 24 hours with PBS, Clys, or IL-17. Supernatants were assessed for CCL20 by ELISA. Data are representative of two independent experiments. All data were analyzed by ANOVA and Student’s t test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Conversely, IL-17 has been shown to induce tissue-protective repair pathways in lung, renal, and intestinal epithelia (38). Therefore, we postulated that IL-17 might offset the cell-damaging effects of candidalysin. We cultured TR146 OECs with live C. albicans or lytic concentrations of candidalysin (70 μM) with or without IL-17 and measured cell damage by lactate dehydrogenase (LDH) activity in supernatants. There was no change in the extent of LDH induced by candidalysin when cells were cultured with IL-17, and as expected, a candidalysin-deficient strain did not induce cell damage (Fig. 7C). Thus, IL-17 neither contributes to nor protects against candidalysin-induced cell damage.

To gain mechanistic insight into signaling cross-talk between IL-17 and candidalysin, we assessed the downstream signaling pathways instigated by these factors. IL-17 activates nuclear factor κB (NF-κB)
among other pathways (39), whereas candidalysin-induced signaling is characterized by p38-MAPK (mitogen-activated protein kinase)/c-Fos activation and phosphorylation of the MKP1 (Dusp1) phosphatase (14, 16). In TR146 cells, treatment with IL-17 induced the phosphorylation of inhibitor of nuclear factor κBα (IkBα), an early step in the canonical NF-κB pathway, albeit more weakly than TNFα (Fig. 7D, left). Candidalysin did not activate the phosphorylation of IkBα nor was there an additive impact of costimulating cells with IL-17 and candidalysin. Although candidalysin stimulated c-Fos up-regulation and phosphorylation of MKP1, there was no synergistic activation of c-Fos or MKP1 in the presence of IL-17 (Fig. 7E). However, knockdown of c-Fos by RNA silencing blocked the synergistic activation of IL-17 and candidalysin (Fig. 7F), confirming the cooperative activation of these pathways. Together, these data support a model in which secretion of candidalysin by C. albicans hyphae during infection induces an innate cytokine response from OECs, which leads to the activation of resident innate TCRβ+ cells through the IL-1 receptor. These innate TCRβ+ cells respond by secreting IL-17, which signals through its receptor on OECs to further amplify the expression of innate immune effector genes in a feed-forward amplification loop, ultimately resulting in the resolution of infection (fig. S7).

**DISCUSSION**

OECs lining the tongue, palate, and buccal mucosa are vital “first responders” to acute microbial infection, and we recently found that IL-17R-dependent signals on keratin-13+ OECs are critical for immunity to oral candidiasis (32). Here, we identified an unexpected mechanism by which OECs orchestrate IL-17-dependent immunity during a primary innate response to OPC. When C. albicans hyphae invade oral epithelial barriers, they secrete the pore-forming peptide candidalysin, which destabilizes membranes and provides access to host cell content and nutrients (16). Candidalysin signaling on OECs prompts the release of IL-1α/β, which drives the proliferation of innate IL-17+ TCRβ+ lymphocytes through both intrinsic and extrinsic mechanisms. In addition, IL-17 synergizes with candidalysin to further enhance proinflammatory signaling, establishing a feed-forward activation loop that mobilizes antifungal host defenses. This scenario ensures that protective IL-17–driven responses only manifest in the presence of tissue-damaging invasion of C. albicans hyphae (fig. S7).

In the mouth, innate TCRβ+ cells and γδ T cells constitute the main early sources of IL-17 (9). Although oral γδ T cells evidently do not proliferate during OPC, their isolation is inefficient, so we cannot rule out the possibility that proliferation in these cells occurs at low levels. Moreover, γδ T cells do contribute to the response; they can express large quantities of IL-17 on a per-cell basis (40), and mice lacking either γδ or αβ T cells exhibit modestly increased susceptibility to OPC, suggesting rudimentary of these cell types (9). Unlike humans, mice are naïve to C. albicans and do not have C. albicans–specific T cells at baseline (7, 8, 41). Our data with Nur77GFP mice (which report ongoing TCR signaling) confirm that the proliferation of the TCRβ+ cells is independent of Candida-specific antigens, at least within the detection limits of this system (Fig. 3). In acute dermal candidiasis, IL-17 is also made by γδ T cells and TCRβ+ cells. However, here, it is the γδ T cells that proliferate and that are comparatively more important than TCRβ+ cells (Fig. 1) (9, 21, 42). Although ILC3s have been implicated in OPC (18), Rag1−/− mice have high fungal loads after oral C. albicans infection (8, 9), and therefore, contributions of this cell type appear to be negligible.

The prevailing paradigm in fungal immunology is that IL-17 responses are triggered upon sensing of hyphal cell wall carbohydrates through dectin/CARD9 or TLR2 signaling (43). Although true for adaptive responses, our data demonstrate that the acute IL-17 response is instead triggered by candidalysin, which is responsible for cellular damage by invasive hyphae. Consequently, the host evidently exploits candidalysin (ECE1) to discriminate between damaging and nondamaging hyphal tissue invasion. Although candidalysin-deficient strains fail to provoke efficient type 17 responses (Fig. 5), strains lacking ECE1 are less virulent in the settings of immunodeficiency (16). Ece1Δ/Δ strains do not persist in immunocompetent mice (16), likely due to mechanical clearance by salivary flow and swallowing. We speculate that in healthy humans where C. albicans is a commensal microbe, nondamaging colonization provides a survival advantage to the fungus because it does not set off host defense alarms through the production of candidalysin. A recent report evaluating different C. albicans strains found a similar, albeit imperfect, correlation of ECE1 levels with IL-17 production (44). C. albicans strains vary in cell wall composition or other properties, so it is possible that in some strains, there are alternate virulence factors that trigger IL-17 responses. Nonetheless, the independent clinical isolate HUN96 (45) induced TCRβ+ proliferation similarly to the CAF2-1 and Bwp17 strains that are more commonly used (both derived from the SC5314 laboratory strain) (fig. S4).

Adaptive T cells with innate properties have been identified in multiple barrier tissues. For example, gingiva-resident CD4+ TCRβ+ IL-17+ cells are induced after mechanical damage upon mastication (46). Like the cells described here, gingival TCRβ+ cells expand by local and rapid proliferation, are activated upon tissue damage, and are present in mice from different vendors. However, these populations differ in their requirements for IL-6 and IL-1, and we previously saw that germ-free mice appear to lack baseline innate TCRβ+ cells in the tongue (9). In the skin, heterologous protection against C. albicans can be conferred by IL-17–secreting CD8+ T cells that are specific for commensal bacteria (47). Similarly, in the eye, γδ T cells specific for an ocular commensal bacterium can provide protection from C. albicans through IL-17 production (48). Innate functions in pulmonary memory Tγδ2 cells that manifest effector responses without engaging the TCR have been described (49), and memory T cells with innate-like functions have been reported in the human mucosa and skin (50). These findings collectively indicate that tissue-resident T cells can be co-opted to function in an innate capacity. It is tempting to speculate that the ability of adaptive cells to function as innate effector cells may be an evolutionary remnant of their role in controlling invasive pathogens at barrier sites.

**MATERIALS AND METHODS**

**Mice**

All mice were on the C57BL/6 background. Experiments were performed on both sexes with age- and sex-matched controls in each experiment. Il17ra−/− mice were provided by Amgen. Nur77GFP mice were from K. Hogquist (University of Minnesota) (23). Card9−/− mice were from X. Lin (MD Anderson Cancer Center). Act1−/− mice were from U. Siebenlist [National Institutes of Health (NIH)] (51). CD4CreIl1r1b+ mice were described (37). Il17aCreRosa26GFP fate reporters (17) and other mouse strains were from JAX (except as noted for Taconic Farms) and housed at the University of Pittsburgh for at least 7 days before experimentation. For adoptive transfers, mice were
irradiated and given $10^6$ femoral BM after 24 hours. Mice were reconstituted for 6 to 9 weeks. Protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. All efforts were made to minimize suffering, in accordance with the Guide for the Care and Use of Laboratory Animals of the NIH.

**Oral candidiasis and *C. albicans* strains**

OPC was induced by sublingual inoculation with a cotton ball saturated in *C. albicans* for 75 min, as described in (5). For rechallenge, mice were infected 6 weeks after the primary infection (8). Tongue homogenates were prepared on a gentleMACS (Miltenyi Biotec), and colony-forming units (CFU) were determined by serial dilutions on YPD agar. Anti–CCL20 (R&D Systems), anti–IL-1α, anti–IL-1β, or isotype control antibodies (Bio X Cell) were administered on day–1 after infection (100 to 1000 μg per mouse). CAF2-1 or Bwp17 *C. albicans* strains (derived from SC5314; www.candidagenome.org/Strains.shtml) were used as WT. Knockout strains (sap4-6Δ/Δ, ece1ΔΔ/Δ, and efg1ΔΔ/Δ) and HUN96 were described (16, 29, 34, 35).

**Flow cytometry**

Flow cytometry was performed as described (9). Tongues were pooled (two per sample), and cell suspensions were prepared with the Tumor Dissociation kit (Miltenyi Biotec). Antibodies were from eBioscience, BD Biosciences, or BioLegend. Proliferation was assessed using the Foxp3/Transcription Factor Buffer kit (eBioscience) with Ki67-APC (BD Pharmingen) or PCNA–PE (phycoerythrin) (eBioscience). Data were acquired on an LSRFortessa and analyzed with FlowJo.

**RNA and qPCR**

Frozen tongue was homogenized in RLT buffer (RNeasy kit; Qiagen). Total RNA was purified using TRIzol reagent (Invitrogen). Relative quantification of gene expression was performed using SYBR Green (Applied Biosystems). Knockdown of c-Fos was performed as described in (14).

**Cell culture, in vitro infections, cytokine stimulations, and immunoblotting**

TR146 cells (ECACC10032305) were cultured in Dulbecco’s modified Eagle Medium (DMEM)–F12/15% fetal bovine serum, as described in (14). For infections in vitro, 3 × 10^5 to 5 × 10^5 cells were seeded over-night and cultured in serum-free DMEM with 1 × 10^5 CFU *C. albicans* yeast cells for 24 hours. Recombinant human IL-17, TNFα, and IL-22 (R&D Systems) were used at 200, 20, or 100 ng/ml, respectively. Candidalysin (SIGIIMGILGNPQVIQIMISSVAKFNGK) was from Peptide Protein Research Ltd. Antibodies used were Phospho-IκBα and IκBα (Upstate Biotechnology), c-Fos and phospho-MKP1 (Cell Signaling), and Actin (clone C4, EMD Millipore).

**Luminex, ELISAs, and LDH assays**

Conditioned medium was analyzed by Luminex (IL-1α, IL-1β, IL-6, GM-CSF, and G-CSF) or ELISA (CCL20) kits from R&D Systems. LDH assays were performed with CytoTox 96 Assay System reagents (Promega).

**Statistics and study design**

Data were analyzed on Prism (GraphPad Software) using ANOVA or Student’s t test. Fungal load data are presented as geometric mean and evaluated by ANOVA with Mann-Whitney correction. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. All experiments were performed a minimum of twice in independently performed replicates.

**SUPPLEMENTARY MATERIALS**

immunology.sciencemag.org/cgi/content/full/2/17/eaam8834/DC1

Fig. S1. TCRβ cell proliferation in knockout mice and gating strategies.

Fig. S2. Expansion of innate TCRβ cells during OPC.

Fig. S3. Baseline frequency of innate TCRαβ cell expansion.

Fig. S4. Virulence factors and TCRαβ+ cell expansion.

Fig. S5. Factors that activate TCRβ+ cell expansion.

Fig. S6. Candidalysin signals synergistically with IL-17 and TNFα but not with IL-22.

Fig. S7. Model of first encounter to *C. albicans* in the oral epithelium.

Data file (provided as Excel file)

Source data (provided as pdf file)

**REFERENCES AND NOTES**


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Oral epithelial cells orchestrate innate type 17 responses to *Candida albicans* through the virulence factor candidalysin


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Calibrating antifungal responses

Immune responses to fungal infections are complicated by the fact that fungi can exist in multiple forms depending on environmental cues. Here, Verma *et al.* have evaluated innate immune responses to *Candida albicans*, a fungus that transitions from yeast to filamentous hyphae as infection progresses. They find that candidalysin, a hypha-associated protein and virulence factor, serves as a danger signal that potentiates the immune response to *C. albicans*. Candidalysin-deficient strains of *C. albicans* caused minimal epithelial damage and elicited a blunted type 17 immune response. The authors propose that the innate antifungal responses to *C. albicans* are driven by a synergy between cellular damage triggered by candidalysin that is further amplified by interleukin-17 driven inflammation.