Oral epithelial cells orchestrate innate Type 17 responses to *Candida albicans* through the virulence factor Candidalysin

Short title: Candidalysin drives innate Type 17 immunity

One Sentence Summary: Innate IL-17 responses to Candida albicans is dependent on Candidalysin, a hypha-associated fungal peptide that drives production of IL-1 in oral epithelial cells.

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Abstract

The dimorphic fungus *Candida albicans* causes severe oral infections in immunodeficient patients. Invasion of *C. albicans* hyphae into oral epithelium is a key virulence trait. Although requirements for innate antifungal immunity remain poorly defined, IL-17 signaling is essential. In mice naïve to *Candida*, IL-17 is produced by several innate lymphocyte subsets, but the only cells that expand upon infection are a poorly understood innate TCRαβ$^+$ population. Here, we show that expansion of these innate TCRαβ$^+$ cells is due to local proliferation, not CCR6/CCL20-dependent recruitment. TCR signaling was not activated during infection, confirming that these TCRαβ$^+$ cells are functionally innate. Activation of innate TCRαβ$^+$ cells did not require signals from conventional PRR signals such as TLR2 or Dectin-1/CARD9. Rather, activation was dependent on the actions of Candidalysin, a hyphal-associated fungal peptide that damages oral epithelial cells (OECs) and triggers the production of DAMPs and inflammatory cytokines including IL-1α/β. Consistently, IL-1R signaling was essential for innate TCRαβ$^+$ proliferation and immunity to OPC, acting in both hematopoietic and non-hematopoietic compartments. Candidalysin-deficient *C. albicans* strains failed to upregulate *Il17a* or stimulate proliferation of innate TCRαβ$^+$ cells. Moreover, Candidalysin signaled synergistically with IL-17 on OECs, further augmenting expression of proinflammatory mediators including IL-1 cytokines. Thus, IL-17 and *C. albicans*, acting through secreted Candidalysin, amplify inflammation in a self-reinforcing feed-forward loop. Our results challenge the current paradigm that hyphal formation per se is required for mucosal innate immunity, but rather demonstrate that establishment of IL-17-mediated antifungal immunity in the oral mucosa requires tissue-damaging invasion of hyphae.

Introduction

Candida albicans is a dimorphic commensal fungus that colonizes human mucosal surfaces. Changes in immune competency or the protective environment of the oral mucosa promote development of oropharyngeal candidiasis (OPC, thrush), a painful opportunistic infection prevalent in settings of HIV/AIDS, iatrogenic immunosuppression, head-neck irradiation, Sjögren's Sydnrome and infancy. Additionally, *C. albicans* is a common cause of vaginal yeast infections, candidemia and disseminated candidiasis; the latter is a frequent hospital acquired infection, with an average 40% mortality rate (*1, 2*). Studies of immunity to fungi have lagged behind other classes of microbes, and to date there are no licensed vaccines for *Candida* species, or indeed to any fungal pathogens. Therefore, understanding the correlates of anti-fungal immunity is essential for informing development of rationale therapeutics and intervention strategies for fungal infections.

The IL-17 signaling pathway is essential for preventing pathogenic *C. albicans* infections (*3*). Patients with IL-17 defects due to mutations in IL-17R signaling pathway components are extremely susceptible to chronic mucocutaneous candidiasis (CMC). Neutralizing antibodies against Th17 cytokines are also associated with CMC, occurring in *AIRE* deficiency and some thymomas (*4*). In this regard, antibodies targeting IL-17 or Th17-related pathways were recently approved for treating psoriasis and other forms of autoimmunity (*5*), and a major infection risk following IL-17 blockade is mucosal candidiasis, especially OPC (*6*). As use of anti-IL-17 biologic drugs expands, the importance of understanding the role of IL-17 in opportunistic infections becomes increasingly compelling.

Similar to humans, mice with IL-17R signaling deficits are susceptible to OPC (*7, 8*). We recently showed that protective IL-17R signaling in OPC is mediated primarily by superficial oral epithelial cell (OECs) (*9*). However, mice do not harbor *C. albicans* as a commensal microbe and are therefore immunologically naïve to this fungus (*10, 11*). Thus, experimental oral *C. albicans* infections in mice most accurately represent the initial encounter to this fungus, which in humans occurs shortly after birth (*3, 12*). Nonetheless, during recall infections with *C. albicans,* mice mount vigorous Th17 responses that significantly augment innate immunity, in keeping with humans where the memory response to *C. albicans* is Th17-dominated (*13*). Consequently, mice provide an ideal model system in which to dissect the requirements for innate 'Type 17' immunity, as distinct from the adaptive Th17 response. In the naïve response,

IL-17 is produced by several innate lymphocyte subsets, but the only cells that expand upon infection belong to an oral-resident innate TCR$\alpha\beta^+$ population, sometimes called 'natural' Th17 cells (*14*).

An essential virulence trait of *C. albicans* is its ability to form invasive hyphae. In systemic candidiasis, the transition to a hyphal state is sensed by a Dectin/Syk/CARD9 signaling cascade in DCs (*15*). Dectin-1 recognizes β-glucan components of the fungal cell wall that are exposed during the hyphal transition, which leads to Th17 cell skewing through DC production of IL-23 and IL-6 (*16, 17*). Surprisingly, neither CARD9 nor IL-6 is required for mounting a primary immune response to OPC (*14, 18*). Therefore, it is unclear how innate Type 17 cells are activated during oral *C. albicans* infection, and why this only occurs in response to invasive, tissue-damaging hyphae.

The initiating event in OPC is exposure of OECs to *C. albicans*. Hyphae but not yeast trigger cell lysis and 'danger' responses in OECs, including production of cytokines (IL-6, IL-1α/β, GM-CSF, G-CSF), antimicrobial peptides (β-defensins) and DAMPs (IL-1α, S100A8/9) (*19, 20*). This OEC activation program is triggered by a newly-discovered fungal virulence factor, Candidalysin (*21*). Candidalysin is a 31-residue amphipathic peptide derived from proteolytic processing of the *ECE1* (Extent of Cell Elongation 1) gene product, and is homologous to pore-forming toxins such as melittin (honeybee) and magainin (African frog). In OECs, Candidalysin triggers a MAPK/c-Fos-dependent cell damage response pathway, concomitant with cellular lysis and upregulation of innate cytokines. Importantly, Candidalysin is secreted only by hyphae yet is not required for hyphal formation (*21*).

Many of the cytokines induced by Candidalysin are associated with Th17 responses or recruitment, e.g., IL-1α/β, IL-6 and CCL20. Given its hypha-specific expression and capacity to induce innate cytokines in OECs, we postulated that Candidalysin might be required for the early IL-17-dependent events following oral *C. albicans* infection. Here we demonstrate that innate oral TCR$\alpha\beta^+$ cells express IL-17 and proliferate in response to *C. albicans* infection without engagement of the TCR or a requirement for signals from Dectin-1, CARD9 or TLR2. Notably, in mice infected with Candidalysin-deficient *C. albicans* strains, innate TCR$\alpha\beta^+$ cells fail to proliferate. This is associated with markedly reduced expression of IL-17, IL-17-dependent genes and IL-1α and IL-1β in the oral mucosa. Consistently, *Il1r1*$^{-/-}$ mice are susceptible to OPC, which was associated with impaired expansion of innate TCR$\alpha\beta^+$ cells and reduced IL-17

expression. Moreover, Candidalysin and IL-17 signal synergistically in OECs to augment expression of antifungal immune response genes, thus amplifying host defense in a feed-forward activation loop. These data reveal that innate IL-17-induced responses are triggered specifically in response to Candidalysin secretion from *C. albicans* hyphae, and illuminate 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$^+$TCRαβ$^+$ cells

We previously showed that IL-17 is induced in the oral mucosa within 24 h of oral infection with *C. albicans* (strain CAF2-1). As the infection is cleared, typically in 3 days, expression of IL-17 concomitantly diminishes (*7, 11*). Using *Il17a^{eYFP}* fate-tracking mice (*22*), we found that IL-17 produced during acute oral *C. albicans* challenge originated dominantly from tongue-resident γδ-T cells and an unconventional population of innate-like CD4$^+$TCRαβ$^+$ cells. Similar findings were reported in *C. albicans*-infected skin (*23*). IL-17 production by ILC3s has also been reported (*24*), though their frequency is below the limit of detection in our hands. These IL-17$^+$TCRαβ$^+$ cells are sometimes termed 'natural' Th17 cells (*14, 25, 26*), but here we refer to them as 'innate TCRαβ$^+$ cells' per Kashem *et al.* (*23*). In the oral cavity, these innate IL-17$^+$TCRαβ$^+$cells reproducibly expand ~2-fold following encounter with *C. albicans*, whereas the frequency of IL-17$^+$γδ-T cells is low and does not change during infection (*14*) (**Fig 1a**). *C. albicans*-dependent expansion of oral innate TCRαβ$^+$ cells was similarly observed in non-fate tracking mice, starting at 1 day p.i. and peaking at day 2 p.i. (**Fig 1b, c, Fig S1**). By day 2, we consistently saw a 2-fold increase in both the percent and total cell number in *C. albicans*-infected mice compared to sham-infected controls harvested within the same experiment (**Fig S1**).

The expansion of innate TCRαβ$^+$ cells could be due to proliferation, survival, recruitment or a combination thereof. To assess proliferation, WT (C57BL/6J) mice were infected orally with *C. albicans* and intracellular Ki67 was measured by flow cytometry. On day 1, Ki67$^+$TCRβ$^+$ cells were more frequent in the infected oral mucosa compared to sham controls (**Fig 1b,** bottom panel). More profound proliferation was observed at day 2 p.i (**Fig 1c,** bottom). Proliferation was confirmed by intracellular staining for proliferating cell nuclear antigen (PCNA) (**Fig 1d**). *C. albicans*-induced proliferation of TCRαβ$^+$ cells was limited to the local site of infection (tongue), as there was no change in the baseline frequency of replicating CD4$^+$TCRαβ$^+$ cells in the draining cervical LN (cLN) (**Fig 1e**). These observations confirm our previous findings demonstrating that IL-17 is detected in the oral mucosa but not in the cLN during a primary *C. albicans* infection (*11*). Notably, the expansion of TCRβ$^+$ cells by *C. albicans* was similar regardless of whether mice were from The Jackson Laboratory or Taconic Farms (**Fig S2a**), and

the proliferating cells exhibited a diverse TCRvβ repertoire (**Fig S2b**). Thus, the 2-fold expansion of TCRαβ$^+$ cells during OPC can be accounted for by local proliferation at the site of infection.

Oral-resident TCRαβ$^+$ cells express CCR6, a marker of Type 17 cells and the receptor for the mucosal-associated chemokine CCL20 (*14*). To determine if signaling through the CCL20/CCR6 axis was required for immunity to oral *C. albicans* infection, we analyzed responses in *Ccr6*$^{-/-}$ mice and mice given neutralizing anti-CCL20 Abs (*27*). Surprisingly, innate TCRαβ$^+$ cells in *Ccr6*$^{-/-}$ mice showed a similar proliferation capacity to WT controls following *C. albicans* infection (**Fig 2a**). There was also no difference in the baseline population of TCRαβ$^+$ cells in the tongue at baseline in *Ccr6*$^{-/-}$ and WT mice (**Fig S3**). Resistance to OPC was similar in *Ccr6*$^{-/-}$ and WT mice, with low oral fungal burdens at 4 days p.i., whereas *Il17ra*$^{-/-}$ controls were susceptible (**Fig 2b**). Similar results were obtained when mice were given anti-CCL20 Abs (**Fig 2c**). Accordingly, the baseline recruitment and *C. albicans*-induced expansion of TCRαβ$^+$ cells in the oral mucosa is independent of CCL20 and CCR6, though we cannot rule out possible involvement of other chemokines.

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

Mice are naïve to *C. albicans*, and animals lacking lymphocytes (e.g., *Rag1*$^{-/-}$, *Il17ra*$^{-/-}$ mice) are highly susceptible to OPC (*11, 14*). To determine whether *C. albicans*-induced expansion of innate TCRβ$^+$ cells requires signals mediated through the TCR, we took advantage of *Nur77*^{eGFP} reporter mice, which report the kinetics and magnitude of TCR signaling by upregulation of the immediate-early gene *Nr4a1* promoter linked to GFP (*28*). As a positive control to verify that TCR activation could be visualized in T cells from the tongue, WT mice were administered agonistic anti-CD3 Abs to activate the TCR in all T cells. This treatment effectively induced GFP in oral TCRβ$^+$ cells (**Fig 3a**). To assess TCR signaling during fungal infection, *Nur77*^{eGFP} mice were challenged orally with *C. albicans* or PBS (sham), and GFP fluorescence in oral TCRβ$^+$ cells was assessed at days 1 and 2 p.i. As expected, T cells from sham-infected mice showed a detectable baseline level of tonic GFP expression (*28*). In mice infected with *C. albicans* for 2 days (1° infection), there was the same baseline GFP fluorescence as seen in sham cohorts, indicating that there was no TCR signaling upon first encounter with

C. albicans and confirming the innate nature of these cells (**Fig 3a**).

The *Nur77*^{eGFP} reporter system can also be used to compare TCR signaling strength, so we assessed the frequency of GFP^{hi} cells (i.e., T cells with more potent TCR signaling) in mice given a primary (1°) or a secondary (2°) *C. albicans* infection. Again, there were no differences between sham-treated mice or those receiving a 2 day (1°) challenge (**Fig 3b**). To verify that *C. albicans*-specific signaling through the TCR could be observed if present, we generated a 2° response by subjecting mice to infection and then re-challenge after 6 weeks; this regimen induces an Ag-specific Th17 response that enhances fungal clearance (*11*). Indeed, there was an increased frequency of GFP^{hi}TCR$\alpha\beta^{+}$ cells in tongues from re-challenged mice, demonstrating that Ag-specific responses can be visualized with *Nur77*^{eGFP} mice in the context of a recall immune response (**Fig 3b**). Therefore, consistent with the naïve state of mice with respect to *C. albicans*, TCR signaling is not activated during expansion of TCR$\alpha\beta^{+}$ cells in a 1° infection.

Pattern recognition receptors required for the adaptive response to C. albicans are dispensable for activation of innate TCR$\alpha\beta^{+}$ cells

Dectin-1 (*Clec7a*) is a C-type lectin pattern recognition receptor used by phagocytes to sense β-glucan carbohydrates that are exposed on *C. albicans* during filamentation. Signaling by Dectin-1 causes APCs to produce IL-23 and IL-6, promoting a Th17 phenotype (*16, 29*). However, it was not known whether Dectin-1 signals would similarly activate innate IL-17-producing cells. In *Clec7a*$^{-/-}$ mice there was rapid and robust proliferation of innate TCR$\alpha\beta^{+}$ cells following oral *C. albicans* infection similar to WT mice, indicating that Dectin-1 is not required for innate TCR$\alpha\beta^{+}$ cell expansion (**Fig 4a**). A similar proliferative response occurred in mice lacking CARD9, a key adaptor downstream of Dectin-1 and other Dectin family members (**Fig 4b**) (*30-32*). TLR2 has also been implicated in innate recognition of *C. albicans* through engagement of hyphae (*29, 33*). However, there was robust proliferation of innate TCRβ^{+} cells in *Tlr2*$^{-/-}$ mice upon 1° *C. albicans*-challenge (**Fig 4c**), indicating that TLR2 is also dispensable.

To determine whether these PRRs were necessary to clear *C. albicans* from the oral cavity during 1° infection, oral fungal loads were assessed 5 days p.i.. Resistance to OPC was not impaired in mice lacking Dectin-1 (**Fig 4d-e**), in agreement with our prior finding that CARD9 is dispensable for innate immunity to OPC (*18*). Similarly, resolution of *C. albicans* infection was not hindered in *Tlr2*$^{-/-}$ mice (**Fig 4d-e**). Hence, TLR2 or Dectin-1 signaling do not

trigger expansion of oral innate TCRαβ$^+$ cells during innate immunity to OPC.

The secreted peptide Candidalysin activates innate TCRαβ$^+$ cell expansion

Hyphal formation is a key virulence trait for *C. albicans*. Consistently, a *C. albicans* mutant that is "locked" in the yeast phase (*efg1Δ/Δ* (*34*)) did not induce *Il17a* expression or expression of downstream genes such as *Defb3, Il1b* or *Ccl20* (**Fig S4a**). In its hyphal state, *C. albicans* secretes a short, amphipathic peptide called Candidalysin, which destabilizes epithelial membranes and triggers OEC production of cytokines such as IL-1α, IL-1β and IL-6 (*21*). Since these cytokines are linked to Type 17 responses (*35*), we postulated that Candidalysin might serve as an activator of innate TCRαβ$^+$ cell expansion and IL-17 production. To test this, *Il17a*^{eYFP} reporter mice were infected with *C. albicans* strains lacking the Candidalysin-encoding gene *ECE1* (*ece1*Δ/Δ) or an *ECE1*-revertant control strain ("Rev"). Mice infected with the *ece1*Δ/Δ strain exhibited markedly reduced expansion of IL-17$^+$TCRαβ$^+$ cells at day 2 p.i. In contrast, *Il17a*^{eYFP} challenged with the *ECE1*-Rev strain showed robust expansion (**Fig 5a**). Similar results were obtained in WT mice (**Fig 5b, c**). The diminished innate TCRαβ$^+$ cell response in *ece1*Δ/Δ-infected mice correlated with reduced proliferation, demonstrated by Ki67 staining (**Fig 5b,** bottom), which was observed at both day 2 and day 3 p.i (**Fig S4b)**. By day 5, the infection was resolved and the T cell proliferative response returned to baseline. Importantly, at day 2 when cells were harvested, fungal loads were comparable, indicating that the altered TCRαβ$^+$ cell proliferation was not due to reduced exposure to fungal components (**Fig 5d**). Therefore, Candidalysin is required for expansion of innate TCRαβ$^+$ cells during acute oral *C. albicans* infection.

Consistent with the reduced expansion of innate TCRαβ$^+$ cells, mice infected with strains lacking *ECE1* or just Candidalysin (ClysΔ/Δ) showed impaired induction of *Il17a* mRNA expression (**Fig 5e**) as well as IL-17-dependent genes, *Defb3* (β-defensin 3) and *S100a9* (**Fig 5f**). Also consistent with these data, neutrophil mobilization to the tongue, regulated in part by IL-17 signaling (*7, 9, 36*), was reduced in *ece1*Δ/Δ-challenged mice (**Fig 5g**). We also verified activation of TCRαβ$^+$ proliferation in response to a different *C. albicans* strain, HUN96 (*37*), a clinical isolate that expresses *ECE1*, induces c-Fos and damages OECs *in vitro* (**Fig S4c**). Thus, Candidalysin triggers an innate IL-17 response in the oral cavity.

C. albicans secretes multiple virulence factors, particularly secreted aspartyl proteinases (*SAP*s). To determine if the innate IL-17 response was specific to Candidalysin, we evaluated TCRαβ$^+$ proliferation following infection with fungal strains lacking the hypha-associated *SAP* genes (*SAP4-6*) (*38, 39*). Strikingly, there was no defect in TCRαβ$^+$ proliferation in response to infection with *sap4-6*Δ/Δ strains 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 impact differentiation or proliferation of some IL-17-producing cells, such as IL-6, IL-1α and IL-1β (*21*). In tongues of mice subjected to 1° *C. 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 (*14*), and here we verified that proliferation of innate TCRαβ$^+$ cells occurred normally in the absence of IL-6 (**Fig S5**). In contrast, there was no expansion or proliferation of oral innate TCRαβ$^+$ cells in infected *Il1r1*$^{-/-}$ mice (**Fig 6b**). 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 (*40*)) (**Fig 6c**). We next used neutralizing Abs 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 non-hematopoietic 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**). Surprisingly, however, regardless of the source of bone marrow, *C. albicans* infection induced TCRαβ$^+$ cell proliferation in both experimental chimera conditions (i.e., WT → *Il1r1*$^{-/-}$ and *Il1r1*$^{-/-}$ → WT). There was some variation in the percent of Ki67$^+$ cells at baseline (Sham) among cohorts, but in all cases there was a clear increase in proliferation after *C. albicans* infection. This result suggests that there are redundant IL-1R-dependent signals in radio-sensitive and radio-resistant

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 bone marrow. 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 (*41*). Again, TCRαβ$^+$ cells proliferated following OPC (**Fig S5c**), indicating that IL-1 signals occur in both hematopoietic and non-hematopoietic cells. 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 signal synergistically and amplify antifungal immunity in OECs

Candidalysin signaling in OECs upregulates inflammatory cytokines such as IL-6, IL-1β, G-CSF and CCL20. Many of these genes are also targets of IL-17 in OECs (*9*). 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 or additively with cytokines such as 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, *ece1*Δ/Δ or Rev) in the presence or absence of IL-17. After 24 h, conditioned supernatants were assessed for Candidalysin-inducible cytokines by Luminex. As shown, there was an additive or synergistic effect of IL-17 with Candidalysin in upregulating cytokines, including IL-1β, IL-6, CCL20 and G-CSF (**Fig 7a**). To determine whether this synergy was mediated by Candidalysin directly, cells were treated with sublytic concentrations of Candidalysin (15 μM) together with IL-17 (200 ng/ml) for 24 h. Indeed, there was a synergistic or additive induction of cytokines in the presence of IL-17 (**Fig 7b**). TNFα showed a 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 (*7*), did not synergize with Candidalysin (**Fig S6**). Thus, IL-17 and TNFα and Candidalysin cooperatively enhance inflammatory signaling in OECs.

Another attribute of Candidalysin is the induction of cell damage, potentially facilitating fungal access to nutrients and invasion into deep tissue (*21*). Conversely, IL-17 has been shown to induce tissue-protective/repair pathways in lung, renal and intestinal epithelia (*5*). Therefore, we postulated that IL-17 might offset the cell-damaging effects of Candidalysin. To address this question, TR146 OECs were cultured with live *C. albicans* or lytic concentrations of Candidalysin (70 μM) with or without IL-17, and cell damage was measured 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**). These data indicate that 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 NF-κB among other pathways (*42*), while Candidalysin-induced signaling is characterized by p38-MAPK/c-Fos activation and phosphorylation of the MKP1 (Dusp1) phosphatase (*19, 21*). In TR146 cells, treatment with IL-17 induced phosphorylation of IκBα, an early step in the canonical NF-κB pathway, albeit more weakly than TNFα (**Fig 7d**, left). Candidalysin did not activate phosphorylation of IκBα, nor was there an additive impact of co-stimulating cells with IL-17 and Candidalysin. While Candidalysin stimulated c-Fos upregulation and phosphorylation of MKP1, there was no synergistic activation of c-Fos or MKP-1 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 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 TCRαβ^{+} cells through the IL-1 receptor. These innate TCRαβ^{+} cells respond by secreting IL-17, which signals through its receptor on OECs to further amplify expression of innate immune effector genes in a feed-forward amplification loop, ultimately resulting in the resolution of infection (**Fig S7**).

Discussion

Mucosal surfaces are continuously exposed to microbes and environmental challenges. In the oral cavity, OECs lining the tongue, palate and buccal mucosa provide an important physical barrier. These cells act as 'first responders' to microbial infection by producing cytokines, chemokines and antimicrobial peptides that mobilize immune defenses or act directly on pathogens. Although not expressed by OECs, IL-17 is a non-redundant anti-*C. albicans* host factor produced by lymphocytes during innate and adaptive responses. IL-17 signaling on OECs is critical for immunity to OPC by stimulating production of β-defensins and recruiting neutrophils (*7, 9, 36*). Here, we have identified a new mechanism by which OECs orchestrate innate Type-17 immunity during oral candidiasis. When *C. albicans* hyphae invade oral epithelial barriers, they secrete the pore-forming peptide Candidalysin, which destabilizes membranes and causes cell damage, thereby potentially providing access to host cell content and nutrients (*21*). Candidalysin activity on OECs prompts the release of cytokines and DAMPs including IL-1α/β, which are necessary to drive proliferation of tongue-resident innate TCRαβ$^+$ IL-17-producing lymphocytes. Moreover, Candidalysin synergizes with IL-17 to further enhance proinflammatory signaling in OECs, thus establishing a feed-forward activation loop to mobilize antifungal host defense. Importantly, this scenario ensures that the protective IL-17-driven responses only manifest in the presence of tissue-damaging invasion of *C. albicans* hyphae (**Fig S7**).

IL-17-dependent responses are pivotal for antifungal immunity in both humans and mice (*3, 4*). In the oral mucosa, innate TCRαβ$^+$ cells and γδ-T cells constitute the main early sources of IL-17 (*14*). We found that IL-17$^+$TCRαβ$^+$ cells undergo rapid expansion during OPC, which was localized at the site of infection. The kinetics of replication and the potent response of oral TCRαβ$^+$ cells are reminiscent of tissue-resident memory T cells described in skin and other mucosal sites (*43, 44*). These oral TCRαβ$^+$ cells are immunologically naïve to *C. albicans* antigens, evidenced by the absence of reactive CD4$^+$ or CD8$^+$ T cells in the draining LN (*10, 11*) and a lack of signaling through the TCR as demonstrated with *Nur77^{GFP}* mice (**Fig 4**). A similar population of innate TCRαβ$^+$ cells was detected in an acute cutaneous candidiasis model (*45*). In contrast to the tongue, dermal γδ-T cells proliferated following *C. albicans* infection and were relatively more important than the innate TCRαβ$^+$ cells in the skin (**Fig 1)** (*14, 45*). Nonetheless, γδ-T cells can express large quantities of IL-17 on a per-cell basis (*46*) and mice lacking either

γδ-T cells or αβ-T cells exhibit increased susceptibility to OPC (*14*). Therefore, both γδ-T cells and innate TCRαβ^{+} cells contribute to innate Type 17 immunity to OPC. Although ILC3s have also been implicated (*24*), *Rag1*$^{-/-}$ mice (which retain ILCs) are highly susceptible to acute OPC (*11, 14*), and therefore contributions of this cell type are apparently negligible.

The prevailing paradigm in fungal immunology is that an IL-17 response is triggered upon sensing of hyphal cell wall carbohydrates through Dectin/Syk/CARD9 and/or TLR2 signaling (*47*). While this model holds for adaptive responses, our data demonstrate this is not the case for oral innate immunity to *C. albicans*. Rather, in OPC the acute IL-17 response is triggered by Candidalysin, a pore-forming secreted toxin. Candidalysin-deficient *C. albicans* strains still form hyphae, adhere to and invade epithelial cells *in vitro* (*21*), but they fail to induce IL-17 efficiently *in vivo* (**Fig 5**). Consequently, the host exploits Candidalysin to discriminate between damaging and non-damaging hyphae and thus contain infection. In agreement with this model, neither Dectin-1 nor TLR2 (recognizing fungal β-glucans and mannans, respectively) was required for acute production of IL-17 or clearance of the fungus. These observations extend our report that CARD9 is dispensable for innate immunity to OPC but required for adaptive Th17 responses (*18*). We did not evaluate TLR4 in this study as it is known to be primarily involved in sensing yeast (*48*). Together, these data reveal distinct differences in activation of innate versus adaptive IL-17-dependent immunity in the oral cavity.

While Candidalysin-deficient *C. albicans* strains fail to induce efficient Type 17 responses (**Fig 5**), strains lacking *ECE1* are less virulent in settings of immunodeficiency (*21*). This seeming paradox results from the fact that Candidalysin is needed for the successful transition to pathogenic infection. *ECE1*-deficient mutants are able to invade epithelial cells in culture, but colonization *in vivo* is more superficial, and they do not persist in immunocompetent mice (*21*) likely due to mechanical clearance by salivary flow and swallowing. We speculate that in healthy humans where *C. albicans* predominantly exists as a commensal, non-invasive colonization of barrier interfaces provides the fungus with a distinct survival advantage, as it does not activate host defense alarms through production of Candidalysin. A recent report evaluating different *C. albicans* strains for their abilities to induce IL-17 in OPC found a similar, though not perfect, correlation of *ECE1* levels with IL-17 production (*49*). *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 can trigger IL-17 responses. Still, we found that the clinical

isolate HUN96 (*50*) induced TCRαβ$^+$ proliferation similarly to CAF2-1 and Bwp17 strains (both derived from the SC5314 lab strain) (**Fig S4**). Additionally, we ruled out a role for hypha-associated *SAP* virulence factors (**Fig S4**).

TCRαβ$^+$ cells with innate properties have been identified at many barrier tissues. In skin, heterologous protection against *C. albicans* can be conferred by IL-17-secreting CD8$^+$ T cells that are specific for commensal bacteria (*52*). A recent report described gingiva-resident CD4$^+$TCRαβ$^+$IL-17$^+$ cells that are induced upon mechanical damage from mastication (*51*). Like the cells described here, gingival TCRαβ$^+$ 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 TCRαβ$^+$ cells in their tongues (*14*). Additionally, innate functions in pulmonary memory Th2 cells have been described that manifest effector responses without engaging the TCR, which depend on IL-33 (*53*). Analogous memory T cells with innate-like functions have also been reported in human mucosae and skin (*54*). These convergent data indicate that tissue-resident TCRαβ$^+$ cells can in some circumstances 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.

The combinatorial signaling of IL-17 and TNFα with Candidalysin was unexpected. IL-17 is a promiscuous activator of inflammation and signals additively or synergistically with numerous cytokines, particularly TNFα (*42*). *In vivo* this property is highly relevant, since inflammatory environments contain multiple stimuli that have the capacity to interact in concert. Although not fully understood, synergy occurs through IL-17-induced transcription factors such as NF-κB and CCAAT/Enhancer binding proteins. IL-17 also extends the half-life of mRNA transcripts that are intrinsically unstable, a common property of cytokine transcripts (*42*). Candidalysin-induced cytokine expression occurs dominantly through the p38 MAPK pathway, particularly c-Fos (*19*), whereas epithelial damage is controlled by the PI3K/Akt pathway (*20*). In contrast, IL-17 is a poor activator of c-Fos and PI3K, and Candidalysin does not appreciably activate NF-κB (**Fig 7**). Thus, the cooperative action of IL-17 and Candidalysin appears to be through independent pathways (c-Fos and NF-κB). Consistently, IL-22, which does not activate NF-κB, did not synergize with Candidalysin (**Fig S6**).

IL-1 is important in directing the innate Type 17 anti-*Candida* response. IL-1R signaling is known to drive proliferation of polarized Th17 cells (*55*), induce IL-17 in γδ-T cells (*46*) and promote adaptive T cell responses to *C. albicans* (*56*). Surprisingly, BM chimeras revealed that IL-1R signaling can be operative in both the radio-sensitive and radio-resistant compartments (**Fig 6**). This result contrasts with the role of IL-1R in lung epithelium during *Aspergillus* infection (*57*), and points to a complex, tissue-dependent activity of this cytokine pathway. We also found a dual contribution of IL-1α and IL-1β in OPC, contrasting with reports of divergent functions for IL-1α and IL-1β in other fungal infections (*57-59*). An unexplored avenue is the Candidalysin-inflammasome-innate T cell axis. IL-1 family cytokines are processed by the inflammasome, and both NLRP3 and NLRC4 have been implicated in immunity to OPC (*33, 60*). Hence, this nexus remains an active area of inquiry.

In summary, our study reveals several attributes of host barrier defenses in the oral cavity, an under-studied mucosal tissue. OECs interact intimately with tissue resident lymphocytes to control invasion of *C. albicans*. Upon detecting a breach in barrier defenses indicated by Candidalysin-induced cell damage, cytokines and DAMPs are released that instigate innate Type-17 immunity, which keeps the dangerous form of this organism in check through expression of antimicrobial peptides and mobilization of neutrophils (**Fig S7**). Since oral candidiasis is a common disease of immunocompromise, it is plausible that this pathway might be harnessed for developing immune-modulatory strategies against microbial infections.

Materials and Methods

Mice

Mice were on the C57BL/6 background. Experiments were performed on both sexes with age- and sex-matched controls. *Il17ra*$^{-/-}$ mice were provided by Amgen (Thousand Oaks, CA). *Nur77*^{GFP} mice were from K. Hogquist (University of Minnesota) (*28*). *Card9*$^{-/-}$ mice were from X. Lin (MD Anderson). *Act1*$^{-/-}$ mice were from U. Siebenlist (NIH) (*61*). CD4^{CRE}*Il1r1*$^{fl/fl}$ mice were described (*41*). *Il17a*^{Cre}*Rosa26*^{eYFP} fate reporters (*22*) and other mice were from JAX (except as noted for Taconic Farms, Albany NY) and housed at the University of Pittsburgh for at least 10 d prior to experimentation. For adoptive transfers, mice were irradiated and given 10^6 femoral BM after 24 h. Mice were reconstituted for 6-9 weeks. Protocols were approved by the University of Pittsburgh IACUC. All efforts were made to minimize suffering, in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH.

Oral Candidiasis

OPC was performed by sublingual inoculation with a 2.5 mg cotton ball saturated in *C. albicans* (CAF2-1) for 75 min, as described (*7*). For re-challenge, mice were infected 6 weeks after the primary infection (*11*). Tongue homogenates were prepared on a GentleMACS (Miltenyi Biotec, Auburn, CA) and CFU determined by serial dilutions on YPD agar. Anti-CCL20 (R&D Systems, Minneapolis, MN), anti-IL-1α, anti-IL-1β or isotype control Abs (BioXCell, West Lebanon, NH) were administered on day -1 p.i. (100-1000 µg/mouse).

Flow Cytometry

Flow cytometry was performed as described (*14*). Tongues were pooled (2 per sample) and cell suspensions prepared with the Tumor Dissociation kit (Miltenyi). Abs were from eBioscience, BD Biosciences or BioLegend. Proliferation was assessed using the Foxp3/Transcription Factor Buffer Kit (eBioscience) with Ki67-APC (BD Pharmigen) or PCNA-PE (eBioscience). Data acquired on an LSR Fortessa and analyzed with FlowJo (Ashland OR).

RNA and qPCR

Frozen tongue was homogenized in RLT buffer (RNAeasy kit; Qiagen) with a GentleMACS Dissociator (Miltenyi). cDNA was synthesized with a SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA). Relative quantification of gene expression was determined by real-time PCR with SYBR green (Quanta BioSciences, Gaithersburg, MD) normalized to *Gapdh*. Primers were from SA Biosciences (Qiagen). Results were analyzed on a 7300 real-time PCR system (Applied Biosystems, Carlsbad, CA). Knockdown of c-Fos was performed as described in (*19*); briefly, $3x10^5$ TR146 cells were serum starved for 24 h, transfected with 37 nM c-Fos siRNA in HiPerFect Reagent (Qiagen) for 2 d. Cells were treated with Clys or IL-17 for 24 h and supernatants analyzed by ELISA.

Cell culture, in vitro infections, cytokine stimulations, immunoblotting

TR146 cells (ECAAC10032305) were cultured in DMEM-F12/15% FBS as described (*19*). For infections *in vitro*, $3\text{-}5x10^5$ cells were seeded overnight and then cultured in serum-free DMEM with $1x10^5$ CFU C. *albicans* yeast cells for 24 h. Recombinant human IL-17, TNFα and IL-22 (R&D Systems) were used at 200, 20 or 100 ng/ml, respectively. Candidalysin peptide (SIIGIIMGILGNIPQVIQIIMSIVKAFKGNK) was from Peptide Protein Research Ltd (UK). Antibodies: Phospho-IκBα and IκBα (Upstate Biotechnology), c-Fos and phospho-MKP1 (Cell Signaling), Actin (clone C4, EMD Millipore).

Luminex, ELISAs and LDH assays

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

Statistics

Data were analyzed on Prism (GraphPad Software), using ANOVA with posthoc Tukey's test, or Student's t test. Fungal loads 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. NS = not significant.

Supplementary Materials

Figure S1. TCR$\alpha\beta^+$ cells reproducibly expand 2-fold following oral infection with *C. albicans*.

Figure S2. Expansion of innate TCR$\alpha\beta^+$ cells during OPC.

Figure S3. Baseline numbers of innate TCR$\alpha\beta^+$ cells are similar in WT and Ccr6$^{-/-}$ mice.

Figure S4. Virulence factors and TCR$\alpha\beta^+$ cell expansion.

Fig S5. Factors that activate TCR$\alpha\beta^+$ cell expansion.

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

Figure S7. Model of Candidalysin-induced immunity to oral candidiasis.

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Abbreviations

Clys, Candidalysin; CMC, chronic mucosal candidiasis; LDH, lactate dehydrogenase; LOD, limit of detection; OEC, oral epithelial cell; OPC, oropharyngeal candidiasis.

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Figure Legends

Figure 1. Proliferation of oral TCRαβ$^+$ cells following *C. albicans* infection. (A) *Il17a*^{eYFP} mice (*22*) were challenged sublingually with PBS (sham) or *C. albicans*. Homogenates were prepared from pooled tongues (n=2). YFP$^+$TCRαβ$^+$ or YFP$^+$TCRγδ$^+$ cells in the CD45$^+$CD4$^+$ gate were assessed by flow cytometry. Data show fold-increase versus sham and analyzed by Student's t-test. Data pooled from 3-4 independent experiments. (B-C) WT mice (C57BL/6J) were infected with *C. albicans,* and tongue homogenates prepared on days 1 or 2 p.i. Cells were gated on lymphocytes and staining of CD45 and TCRβ is shown (top). Proliferation of CD45$^+$CD4$^+$TCRβ$^+$ cells was determined by staining for Ki67 (bottom). Data representative of 15 experiments. Graph C: mean ± SEM of proliferating TCRαβ$^+$ cells on days 1 and 2, analyzed by Student's t-test. (D) WT mice were infected with *C. albicans* and tongue homogenates prepared on day 2 p.i. Proliferation was determined by PCNA staining. Data representative of 3 experiments. (E) WT cervical LNs were harvested on day 2 p.i. Proliferation of CD45$^+$CD4$^+$TCRβ$^+$ cells was determined by anti-Ki67 staining. Graph shows mean ± SEM of Ki67$^+$ CD4$^+$ cells in cLNs, analyzed by Student's t-test. Data are representative of 2 experiments.

Figure 2. CCR6 is dispensable for expansion of innate TCRαβ$^+$ cells in oral candidiasis. (A) WT or *Ccr6*$^{-/-}$ mice were infected with *C. albicans,* and tongue homogenates prepared 2 days p.i. Proliferation of TCRαβ$^+$ cells was determined by anti-Ki67 staining. Data representative of 3 independent experiments. (B) Indicated mice were infected orally with *C. albicans,* and fungal burden was assessed by CFU enumeration on day 4 p.i.. Bars = geometric mean. Each point represents an individual mouse. Dashed line = limit of detection (LOD, 30 CFU) (*62*). Data are compiled from 4 independent experiments. Data analyzed by ANOVA with Mann-Whitney correction. (C) WT mice were injected with 100 ug anti-CCL20 Abs or isotype controls on day -1 relative to infection. Mice were infected with *C. albicans,* and proliferation of TCRαβ$^+$ cells was determined by anti-Ki67 staining 2 days p.i. Data are representative of 2 experiments.

Figure 3. A primary *C. albicans* infection activates innate TCRαβ$^+$ cells without engaging the TCR. (A) *Nur77*^{GFP} mice were sham-treated (red line) or infected with *C. albicans* and tongue homogenates prepared on days 1 or 2 p.i (blue line). Controls were given agonistic anti-

CD3 Abs (green line) to stimulate the TCR on all T cells. WT ("non-Tg") mice were negative controls for GFP staining (grey line). Left: fluorescence intensity of GFP in oral $CD45^+CD4^+TCR\beta^+$ cells was assessed by flow cytometry. Right: Relative mean fluorescence intensity (MFI) of GFP in $CD45^+CD4^+TCR\beta^+$ cells was assessed and normalized to sham. NS, not significant. Data are from 3 independent experiments. (B) *Nur77*^{GFP} mice were infected with *C. albicans*. Tongue homogenates were prepared 2 d p.i. ("1° Inf"). In order to induce *C. albicans*-specific TCR signaling ("2° Inf"), mice were infected orally, rested for 6 weeks, and then re-challenged with a second oral infection, as described (*11*). Left: GFP fluorescence in oral $CD45^+CD4^+TCR\beta^+$ cells, with % GFP^{hi} cells indicated. Green line shows staining in mice administered agonistic anti-CD3 Abs, as in panel A. Right: Compiled percentage of GFP^{hi} cells in each cohort. Data representative of 3-4 independent experiments. Graphs show mean + SEM, analyzed by ANOVA and student's t-test.

Figure 4. TLR2 and Dectin-1 are dispensable for *C. albicans*-induced proliferation of innate TCRαβ$^+$ cells. (A-C) Indicated mice were infected with *C. albicans* and tongue homogenates prepared 2 days p.i. Proliferation of $CD45^+CD4^+TCR\beta^+$ cells was determined by anti-Ki67 staining. Data representative of 2-3 independent experiments. (D) Indicated mice were infected with *C. albicans,* and fungal burden quantified on day 5 p.i. Bars = geometric mean. Dashed line = LOD. Data from 2 independent experiments. Data analyzed by ANOVA, Mann Whitney correction. (E) Average % weight loss is shown.

Figure 5. Candidalysin drives proliferation of innate IL-17-producing TCRαβ$^+$ cells.
(A) *Il17a*^{eYFP} mice were infected with *C. albicans* (*ece1*Δ/Δ or Revertant "Rev") and homogenates prepared 2 d p.i. Staining of CD45 and YFP in lymphocyte gate is shown. Data representative of 2 experiments. (B) WT mice were infected with the indicated strains of *C. albicans* and homogenates prepared 2 days p.i. Staining of CD45 and TCRβ in lymphocyte gate is shown (top). Proliferation of $TCR\alpha\beta^+$ cells was determined by Ki67 staining (bottom). Data representative of 3 experiments. (C) Fold-expansion of $TCR\beta^+$ cells following infection with *ece1*Δ/Δ or Rev strains. Data pooled from 4 experiments. (D) Fungal loads were assessed at day 2 p.i.. Bar = geometric mean. Data were analyzed by t-test with Mann-Whitney correction and are pooled from 2 experiments. (E-F) Tongue homogenates were prepared on day 2 p.i.

following infection with the indicated *C. albicans* strains. Total mRNA was subjected to qPCR for the indicated genes normalized to *Gapdh*, analyzed by ANOVA and student's t-test. Graphs show mean + SEM normalized to sham. Data compiled from 7-8 mice per group from 2 experiments. (G) Percentage of $CD11b^{+}Ly6G^{hi}$ cells in tongue was assessed at day 2 p.i. + SEM. Data analyzed by ANOVA and student's t-test, from 3 experiments.

Figure 6. IL-1 activates innate TCRαβ^{+} T cell proliferation and anti-fungal immunity in a T cell intrinsic and T cell extrinsic manner. (A) WT mice were infected with the indicated strains of *C. albicans* and tongue homogenates prepared on day 2 p.i. Total mRNA was subjected to qPCR, relative to *Gapdh*, analyzed by ANOVA and student's t-test. Data show mean + SEM normalized to sham. Data from 7-8 mice/group from 2 experiments. (B) Expansion of TCRαβ^{+} cells in *Il1r1*$^{-/-}$ mice 2 days p.i. Proliferation of TCRαβ^{+} cells in the oral lymphocyte gate was determined by Ki67 staining. Data pooled from 3 experiments. (C) Fungal burdens in the indicated mice were quantified on day 5 p.i. Bars = geometric mean. Each point represents 1 mouse. Dashed line = LOD. Data pooled from 2 experiments, analyzed by ANOVA with Mann-Whitney correction. (D) WT mice were administered anti-IL-1α, anti-IL-1β or isotype control Abs (1.0 mg/mouse used alone or 0.5 mg each when used together) on day -1 p.i.. Proliferation of oral TCRαβ^{+} cells was determined by Ki67 staining 2 days p.i. Data representative of 2 experiments. (E) Reciprocal adoptive transfers of femoral BM were performed in WT or *Il1r1*$^{-/-}$ mice. Proliferation of oral TCRαβ^{+} cells was determined by Ki67 staining 2 days p.i. Experimental chimera results are representative of 2 experiments; control chimera data are from 1 experiment.

Figure 7. Candidalysin and IL-17 signal synergistically or additively in oral epithelial cells. (A) TR146 OECs were untreated ("U", grey bars) or stimulated with IL-17 (200 ng/ml, black bars). Cells were infected with WT *C. albicans* (Bwp17+CIp30, "Parent"), *ece1*Δ/Δ or the Revertant (Rev) for 24 h. Conditioned supernatants were analyzed by Luminex (IL-1β, IL-6, G-CSF) or ELISA (CCL20). Graphs indicate mean + SEM. Data analyzed by ANOVA and Student's t-test, representative of 2 experiments. (B) TR146 cells were untreated ("U", grey bars) or treated with IL-17 (200 ng/ml, black bars) or Candidalysin peptide (Clys, 15 μM) for 24 h. Supernatants were analyzed by Luminex (IL-1β, IL-6, G-CSF) or ELISA (CCL20). Graphs

indicate mean + SEM, analyzed by ANOVA and student's t-test. (C) TR146 cells were incubated with *C. albicans* ± IL-17 (200 ng/ml). LDH in supernatants was evaluated after 24 h, representative of 3 experiments. (D) TR146 cells were incubated with TNFα (20 ng/ml), IL-17 (200 ng/ml) or Candidalysin (15 μM) for 5 min. Lysates were immunoblotted for phospho-IκBα or total IκBα. (E) TR146 cells were incubated with TNFα (20 ng/ml), IL-17 (200 ng/ml) or Candidalysin (15 μM) for 30 min or 2 h. Lysates were immunoblotted for c-Fos, phospho-MKP1 or Actin. Data are representative of 2 experiments. (F) TR146 cells were transfected with c-Fos siRNA and stimulated for 24 h with PBS, Clys or IL-17. Supernatants were assessed for CCL20 by ELISA. Data are representative of 2 independent experiments.

Figure 1

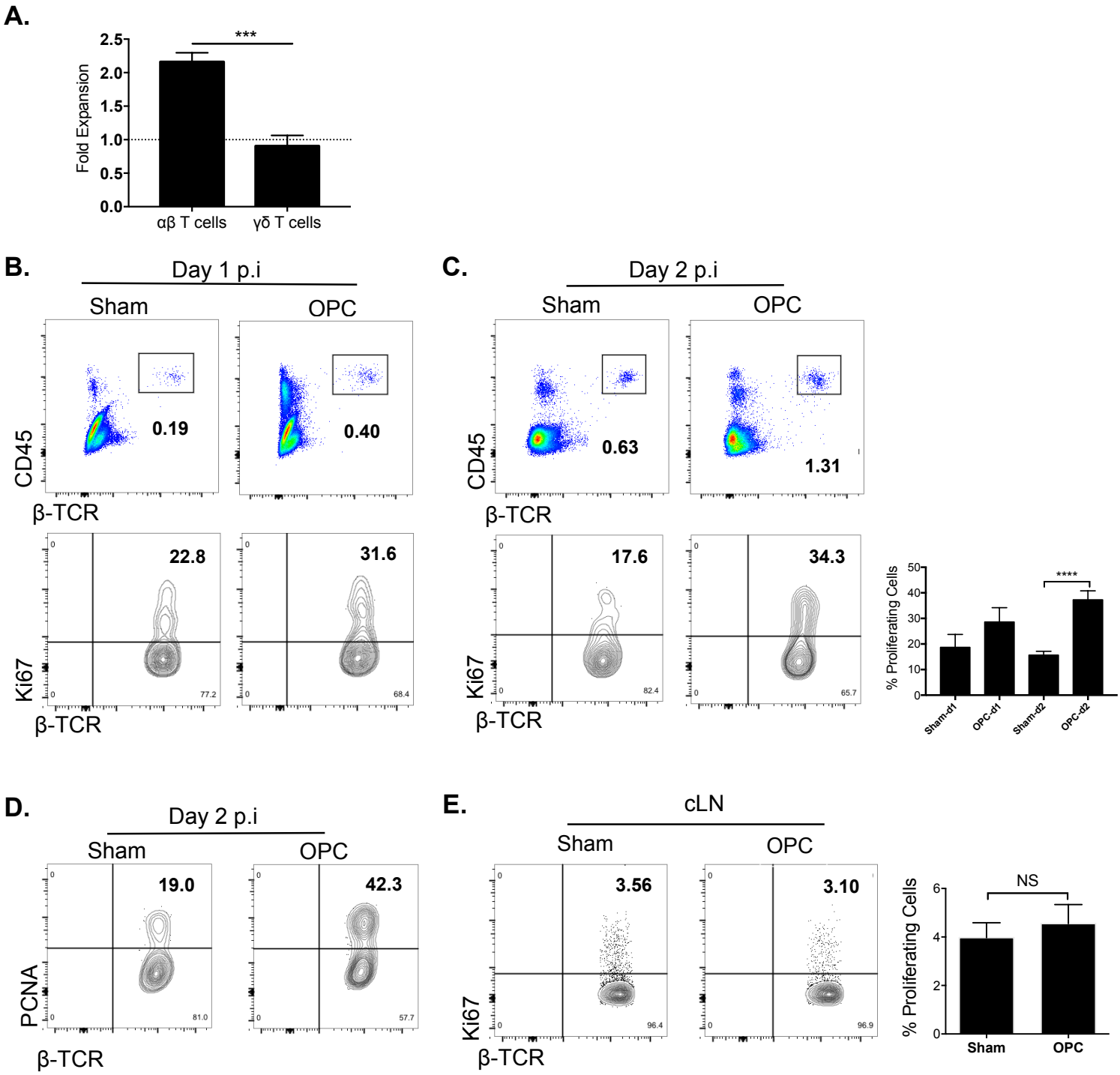

A.
***
Fold Expansion
2.5
2.0
1.5
1.0
0.5
0.0
αβ T cells
γδ T cells
B.
Day 1 p.i
Sham
OPC
CD45
β-TCR
0.19
0.40
Ki67
β-TCR
22.8
31.6
77.2
68.4
C.
Day 2 p.i
Sham
OPC
CD45
β-TCR
0.63
1.31
Ki67
β-TCR
17.6
34.3
82.4
65.7
% Proliferating Cells
****
Sham-d1
OPC-d1
Sham-d2
OPC-d2
D.
Day 2 p.i
Sham
OPC
PCNA
β-TCR
19.0
42.3
81.0
57.7
E.
cLN
Sham
OPC
Ki67
β-TCR
3.56
3.10
96.4
96.9
% Proliferating Cells
NS
Sham
OPC

Figure 2

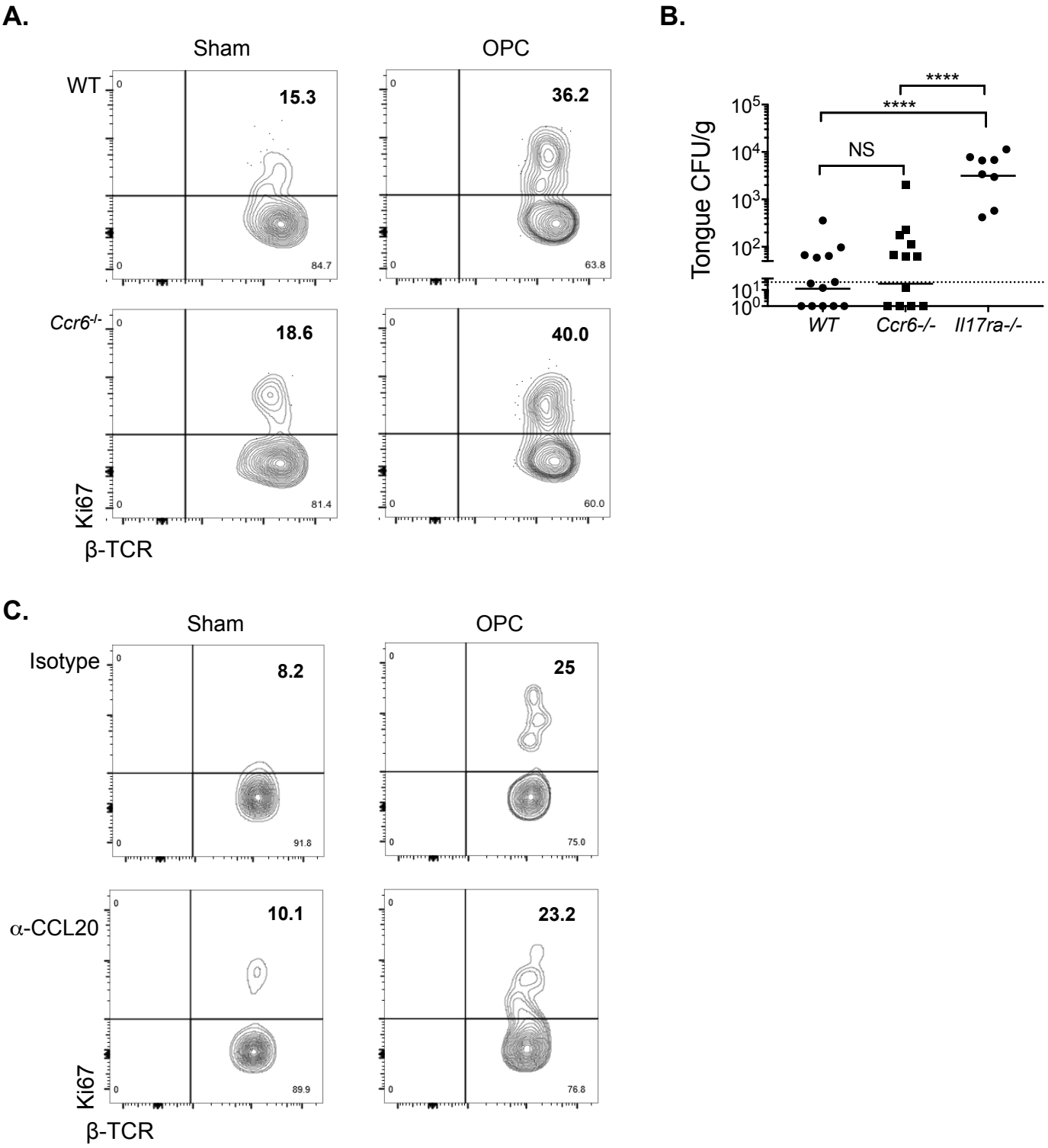
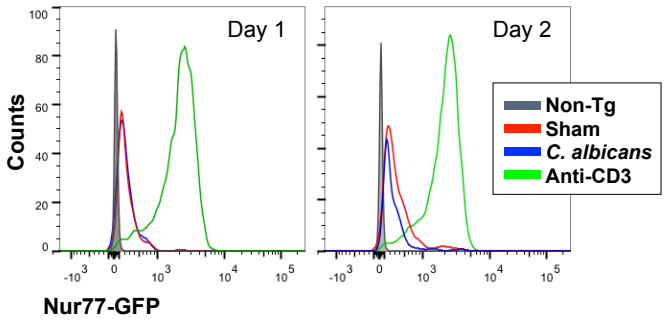
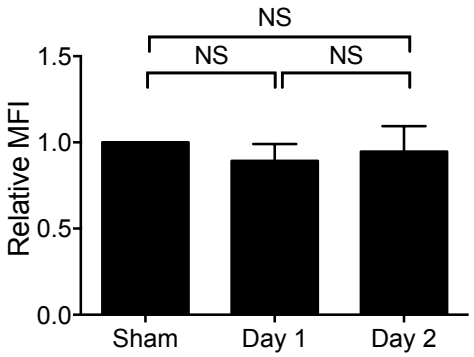

A.
Sham
OPC
WT
15.3
36.2
84.7
63.8
Ccr6-/-
18.6
40.0
81.4
60.0
Ki67
β-TCR
B.
Tongue CFU/g
****
****
NS
WT
Ccr6-/-
Il17ra-/-
C.
Sham
OPC
Isotype
8.2
25
91.8
75.0
α-CCL20
10.1
23.2
89.9
76.8
Ki67
β-TCR

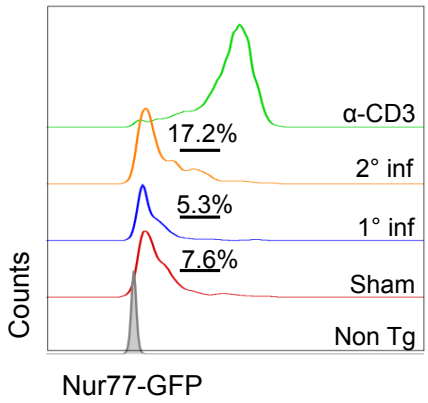
Figure 3

A.


Day 1
Day 2
Counts
Non-Tg
Sham
C. albicans
Anti-CD3
Nur77-GFP


NS
NS
NS
Relative MFI
1.5
1.0
0.5
0.0
Sham
Day 1
Day 2

B.


α-CD3
17.2%
2° inf
5.3%
1° inf
7.6%
Sham
Non Tg
Counts
Nur77-GFP

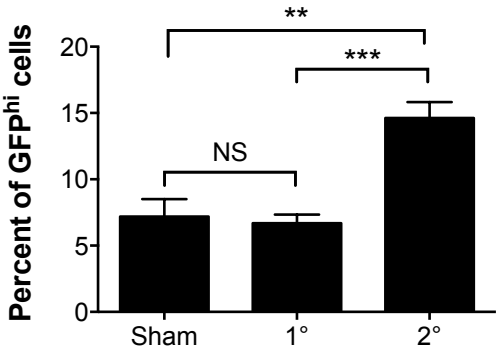

**
***
NS
Percent of GFPhi cells
20
15
10
5
0
Sham
1°
2°

Figure 4

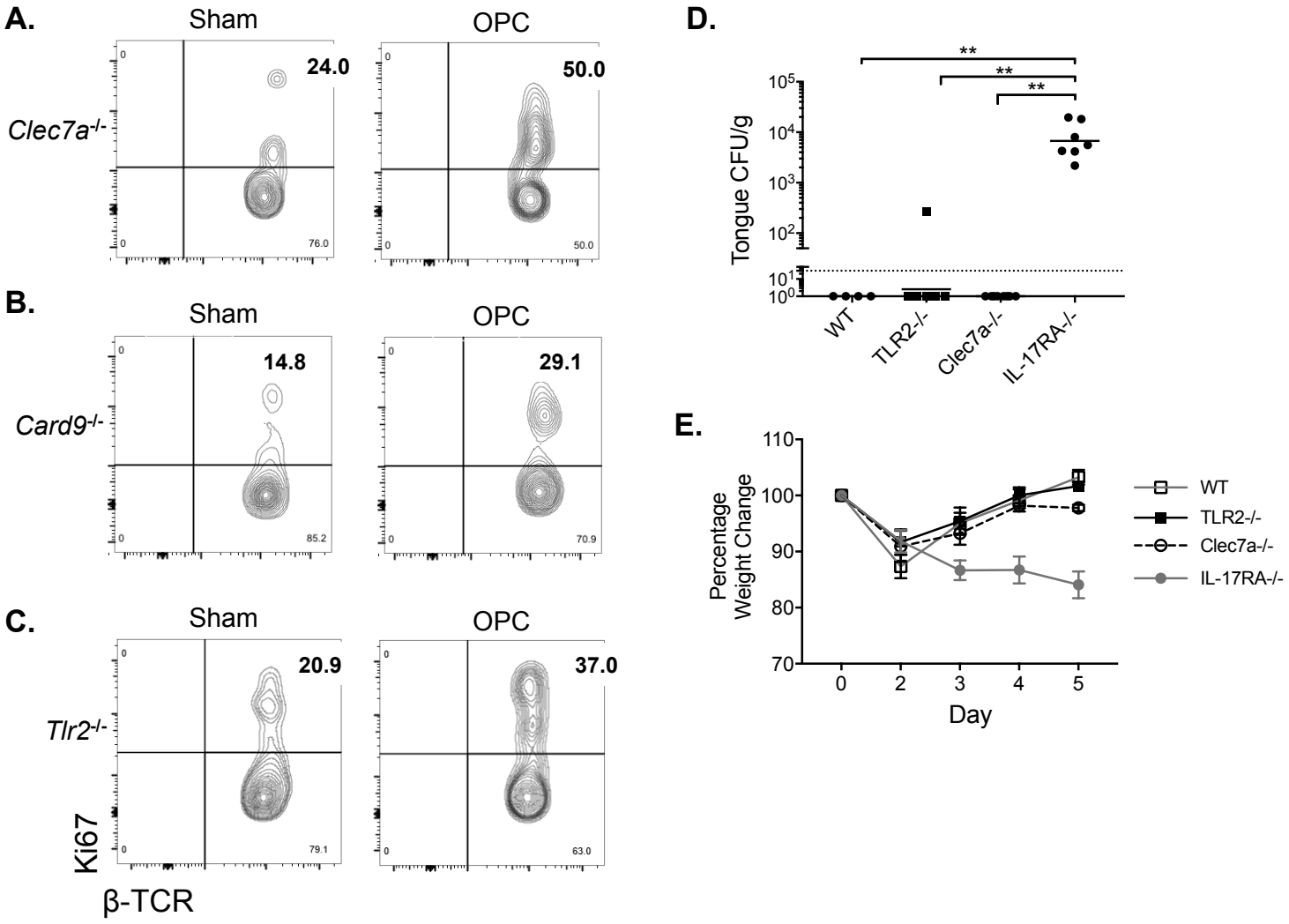

A.
Sham
OPC
Clec7a-/-
24.0
76.0
50.0
50.0
B.
Sham
OPC
Card9-/-
14.8
85.2
29.1
70.9
C.
Sham
OPC
Tlr2-/-
20.9
79.1
37.0
63.0
Ki67
β-TCR
D.
Tongue CFU/g
WT
TLR2-/-
Clec7a-/-
IL-17RA-/-
**
**
**
E.
Percentage Weight Change
Day
WT
TLR2-/-
Clec7a-/-
IL-17RA-/-

Figure 5

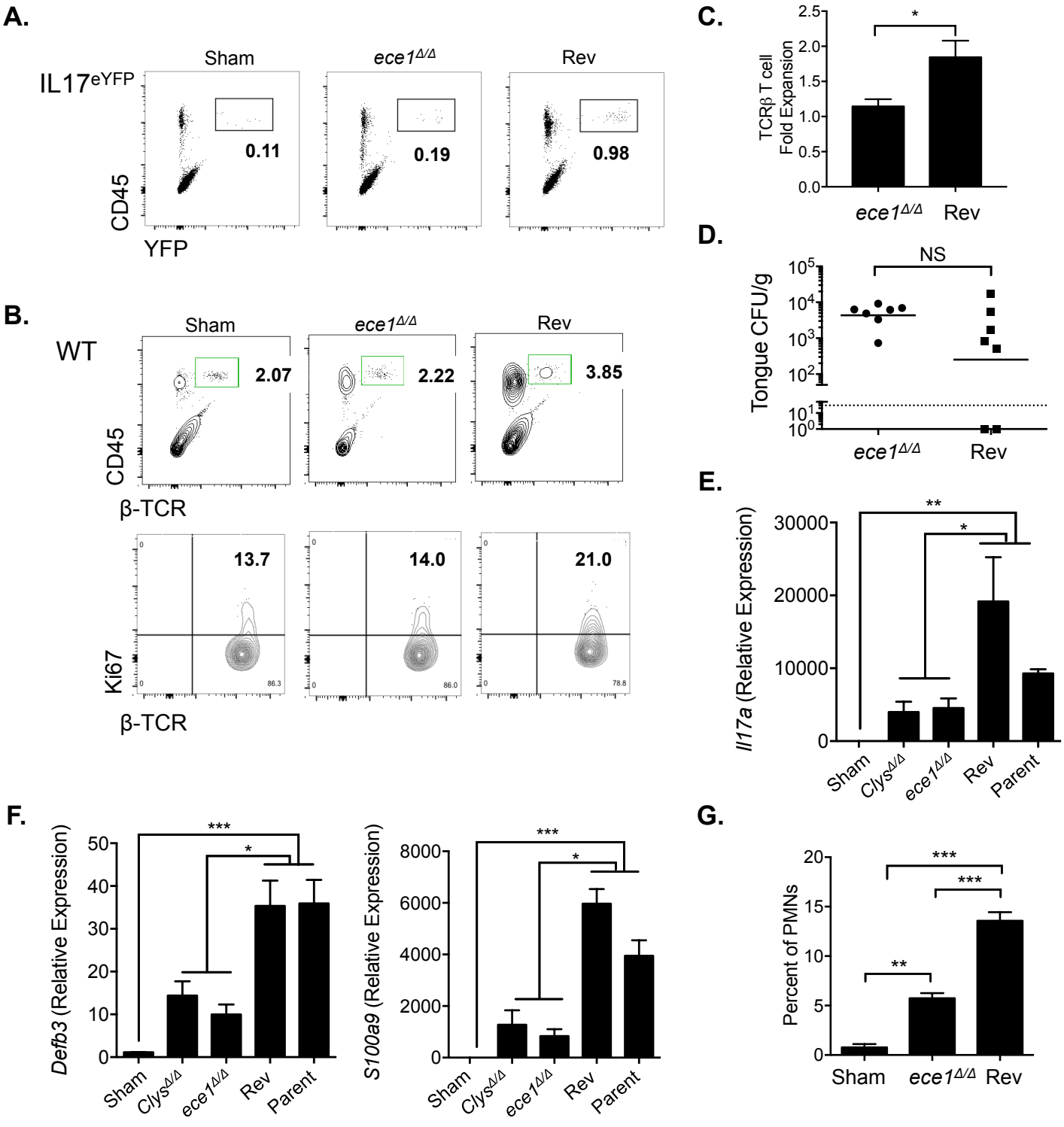
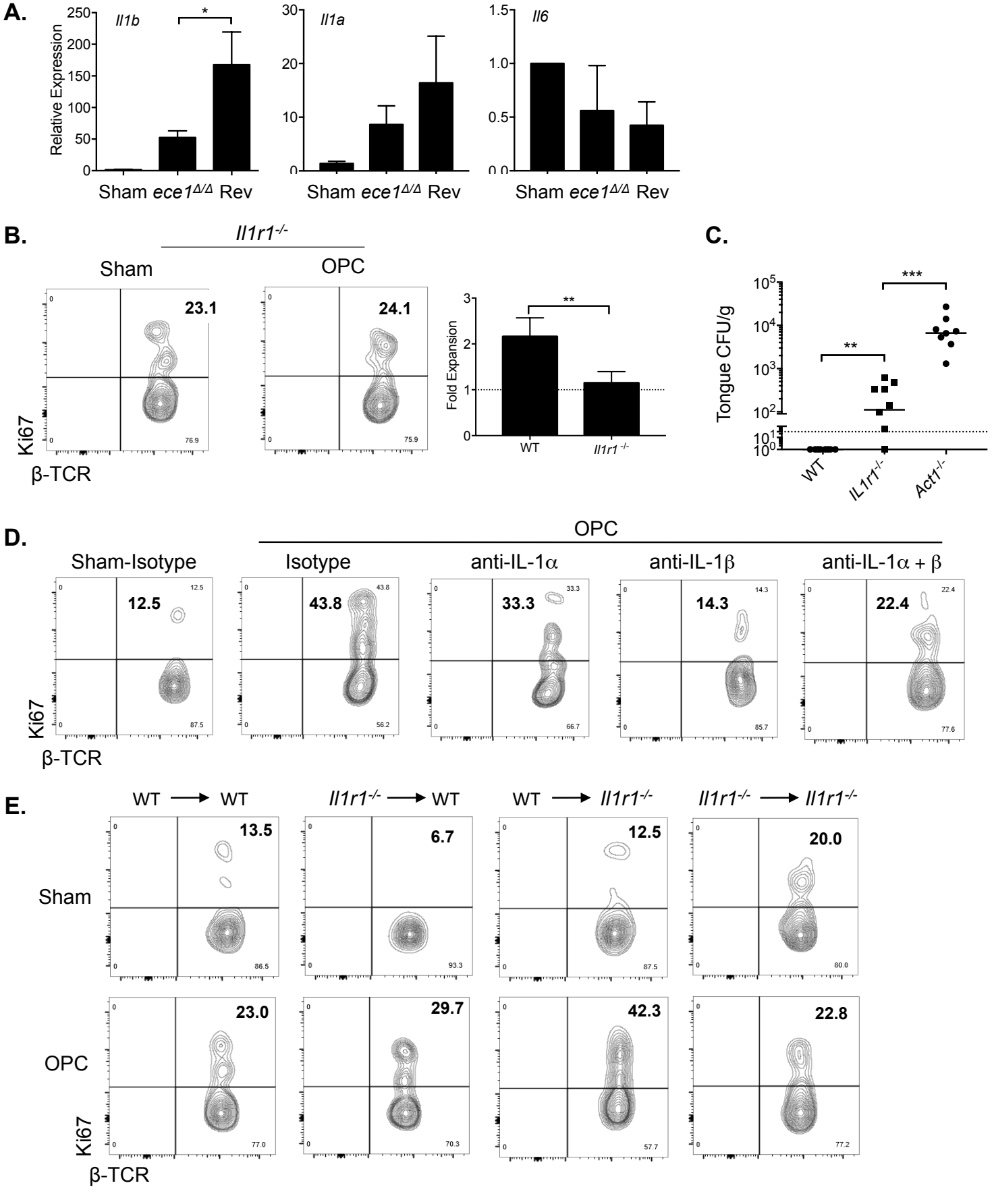

A.
Sham
ece1Δ/Δ
Rev
IL17eYFP
0.11
0.19
0.98
CD45
YFP
B.
WT
Sham
ece1Δ/Δ
Rev
2.07
2.22
3.85
CD45
β-TCR
13.7
14.0
21.0
Ki67
β-TCR
C.
TCRβ T cell Fold Expansion
ece1Δ/Δ
Rev
D.
NS
Tongue CFU/g
ece1Δ/Δ
Rev
E.
Il17a (Relative Expression)
Sham
Clys Δ/Δ
ece1Δ/Δ
Rev
Parent
F.
Defb3 (Relative Expression)
S100a9 (Relative Expression)
Sham
Clys Δ/Δ
ece1Δ/Δ
Rev
Parent
G.
Percent of PMNs
Sham
ece1Δ/Δ
Rev

Figure 6


A.
Il1b
Il1a
Il6
Relative Expression
Sham ece1Δ/Δ Rev
B.
Il1r1-/-
Sham
OPC
23.1
24.1
76.9
75.9
Ki67
β-TCR
Fold Expansion
WT
Il1r1 -/-
C.
Tongue CFU/g
WT
IL1r1-/-
Act1-/-
D.
OPC
Sham-Isotype
Isotype
anti-IL-1α
anti-IL-1β
anti-IL-1α + β
12.5
43.8
33.3
14.3
22.4
87.5
56.2
66.7
85.7
77.6
E.
WT → WT
Il1r1-/- → WT
WT → Il1r1-/-
Il1r1-/- → Il1r1-/-
Sham
OPC
13.5
6.7
12.5
20.0
86.5
93.3
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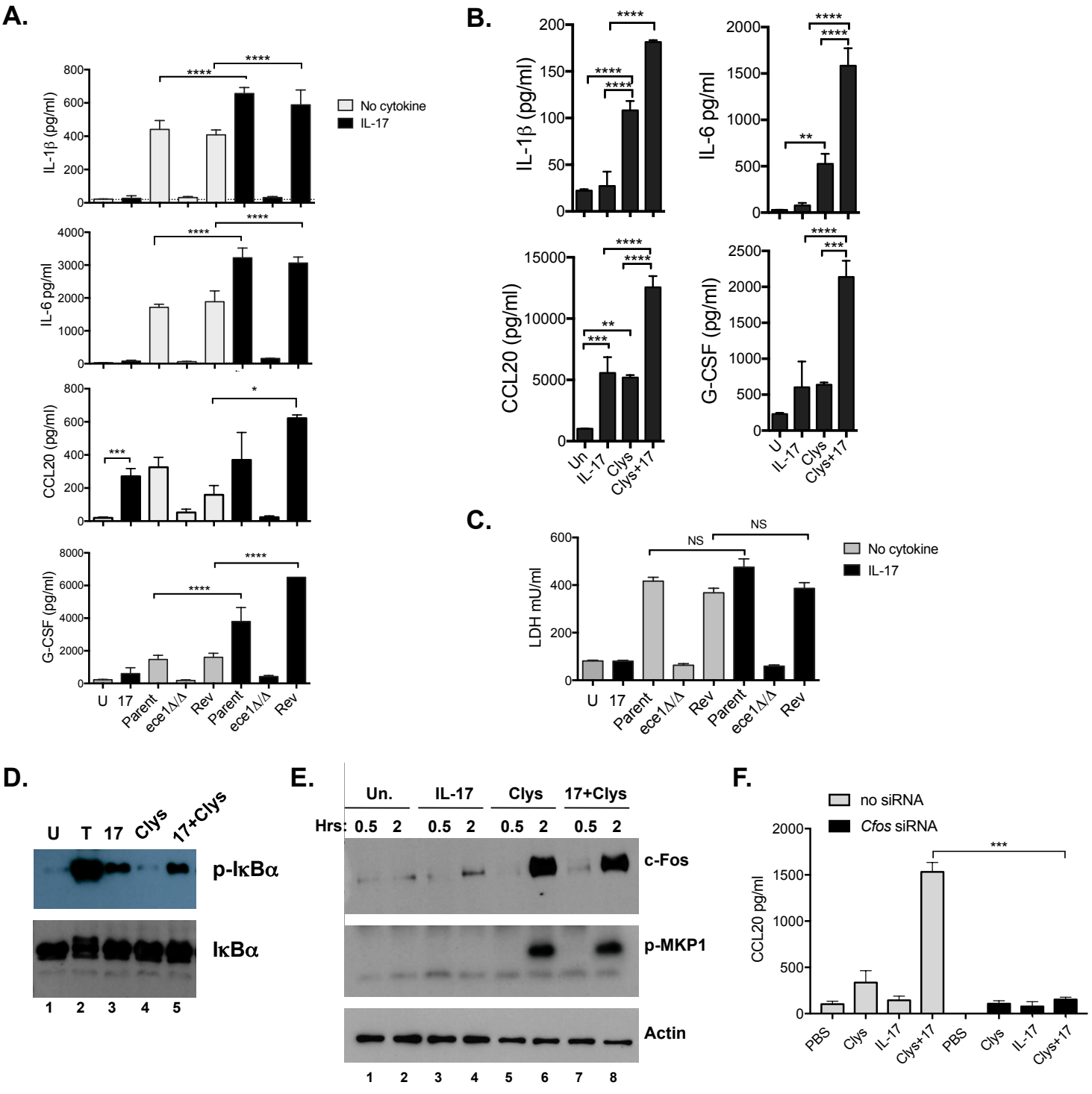
80.0
23.0
29.7
42.3
22.8
77.0
70.3
57.7
77.2

Figure 7


A.
IL-1β (pg/ml)
IL-6 pg/ml
CCL20 (pg/ml)
G-CSF (pg/ml)
No cytokine
IL-17
U
17
Parent
ece1Δ/Δ
Rev
B.
IL-1β (pg/ml)
IL-6 pg/ml
CCL20 (pg/ml)
G-CSF (pg/ml)
Un
IL-17
Clys
Clys+17
C.
LDH mU/ml
NS
No cytokine
IL-17
D.
U
T
17
Clys
17+Clys
p-IκBα
IκBα
1 2 3 4 5
E.
Un.
IL-17
Clys
17+Clys
Hrs: 0.5 2 0.5 2 0.5 2 0.5 2
c-Fos
p-MKP1
Actin
1 2 3 4 5 6 7 8
F.
no siRNA
Cfos siRNA
CCL20 pg/ml
PBS
Clys
IL-17
Clys+17

Supplementary Figures (Verma et al.)

Figure S1. **TCRαβ$^+$ cells reproducibly expand 2-fold following oral infection with *C. albicans*.** WT mice (C57BL/6J) were infected and tongue homogenates prepared on days 1 or 2 p.i. Proliferation of oral TCRαβ$^+$ cells (CD45$^+$CD4$^+$TCRβ$^+$) was determined by intracellular staining with anti-Ki67 Abs. Average percent of Ki67$^+$ cells in Sham samples compared to matched *C. albicans*-infected samples ("OPC") analyzed in the same experiment are indicated by connecting lines. Data are compiled from 2-4 independent experiments. ****P<0.0001 by Student's t-test. NS, not significant.

Figure S2. Expansion of innate TCRαβ$^+$ cells during OPC. A. Expansion of innate TCRαβ$^+$ cells occurs in mice from different vendors. C57BL/6 mice from The Jackson Laboratory (JAX) or Taconic Farms (Tac) were infected orally with *C. albicans* (strain CAF2-1) and after 2 days p.i. tongue homogenates were stained for CD45, CD4, TCRβ and intracellular Ki67. Data are representative of 2 independent experiments. **B. Distribution of TCRvβ expansion during OPC.** WT mice were infected and tongue homogenates prepared on day 2 p.i. Proliferating oral TCRαβ$^+$ cells (Ki67$^+$) were stained for the indicated TCRvβ chains. Percentage of each subtype was assessed. Data are representative of 2 independent experiments.

Figure S3. Baseline numbers of innate TCRαβ$^+$ cells are similar in WT and Ccr6$^{-/-}$ mice. The indicated mice were treated with PBS ("sham") and tongue homogenates prepared on day 2 p.i. Cells were stained for CD45, CD4 and TCRβ. Data are representative of 3 independent experiments.

Figure S4. Virulence factors and TCRαβ$^+$ cell expansion. A. Yeast-locked *Candida albicans* strains do not induce inflammatory responses in oral candidiasis. WT mice were infected with *efg1Δ/Δ* (*37*) or WT strains of *C. albicans* and tongue homogenates were prepared on day 2 p.i. Total RNA was analyzed for the indicated genes by qPCR, normalized to *Gapdh*. Bars indicate mean + SEM. N=3-4 mice per group. Data are from 2 experiments. **B. Expansion of TCRαβ$^+$ cells is impaired at day 3 in response to**

***C. albicans* strains lacking *ECE1*.** WT mice were infected with the indicated strains of *C. albicans* and tongue homogenates were prepared on day 3 p.i. Proliferation of oral TCRαβ$^+$ cells (CD45$^+$CD4$^+$TCRβ$^+$) was determined by intracellular staining with anti-Ki67 Abs. Data are representative of 2 independent experiments. **C. The HUN96 strain of *C. albicans* triggers proliferation of oral TCRαβ$^+$ cells.** Left: WT mice were infected with *C. albicans* HUN96 and tongue homogenates were prepared on day 2 p.i. Proliferation of oral TCRαβ$^+$ cells (CD45$^+$CD4$^+$TCRβ$^+$) was determined by intracellular staining with anti-Ki67 Abs. Right: TR146 cells were incubated with the indicated *C. albicans* strains for 2 h (MOI=10) and expression of c-Fos, phospho-MKP1 and α-actin were assessed by immunoblotting. Note: All data are from the same gel. Data are representative of 2 independent experiments. **D. *SAP4-6* proteases are not required for induction of TCRαβ$^+$ T cell expansion.** WT mice were infected with the indicated strains of *C. albicans* and tongue homogenates were prepared on day 2 p.i. Proliferation of oral TCRαβ$^+$ cells (CD45$^+$CD4$^+$TCRβ$^+$) was determined by intracellular staining with anti-Ki67 Abs. Data are representative of 2 independent experiments.

Fig S5. Factors that activate TCRαβ$^+$ cell expansion. A. IL-6 is not required for expansion of TCRαβ$^+$ cells. WT or *Il6*$^{-/-}$ mice were infected with *C. albicans* and tongue homogenates were prepared on day 2 p.i. Proliferation of oral TCRαβ$^+$ cells (CD45$^+$CD4$^+$TCRβ$^+$) was determined by intracellular staining with anti-Ki67 Abs. Data are representative of 2 independent experiments. **B. Mixed BM chimeras indicate that there is not a T cell intrinsic requirement for IL-1R-driven T cell proliferation in the context of a WT host.** WT mice were irradiated and 24 h later reconstituted with mixed BM from WT (CD45.1) and *Il1r1*$^{-/-}$ (CD45.2) mice in a 50:50 ratio. After 8 weeks of reconstitution, mice were infected orally with *C. albicans* and tongue homogenates were prepared on day 2 p.i. Left: FACS plots showing percentage of congenically marked CD45$^+$CD4$^+$TCRβ$^+$ cells in tongue. Right: Proliferation of oral CD45.2 (*Il1r1*$^{-/-}$) or CD45.1 (WT) TCRαβ$^+$ cells after infection. Data are representative of 2 independent experiments **C. Mice lacking IL-1R on CD4$^+$ cells do not show impaired T cell proliferation after *C. albicans* infection.** WT mice (CD45.1) were irradiated and 24 h later reconstituted with BM from CD4^{CRE}*Il1r1*$^{fl/fl}$ (CD45.2) mice. After 8 weeks of

reconstitution, mice were infected orally with *C. albicans*, and proliferation of CD45.2 (donor) TCRβ^+ cells was assessed by Ki67 staining. Data are representative of 2 experiments.

Fig S6. Candidalysin signals synergistically with IL-17 and TNFα but not IL-22. TR146 cells were untreated ("U") or treated with IL-17 (200 ng/ml), TNFα (20 ng/ml), IL-22 (100 ng/ml) or a Candidalysin peptide (Clys, 15 μM) as indicated for 24 h. Conditioned supernatants were analyzed by Luminex. Graphs indicate mean + SEM, analyzed by ANOVA and student's t-test. Data are representative of 2 independent experiments.

Figure S7. Model of Candidalysin-induced immunity to oral candidiasis. When *C. albicans* undergoes morphotype switching to an invasive hyphal form, Candidalysin is secreted which damages the oral epithelium. This triggers the activation of c-Fos via MAPK signaling and the production of cytokines such as IL-1α and IL-1β. IL-1 induces the proliferation of resident innate TCRαβ$^+$ cells through signals in both the hematopoietic and non-hematopoietic compartments, and these innate T cells in turn secrete IL-17. IL-17 induces NF-κB-dependent signals in the oral epithelium that further amplify production of IL-1 and other inflammatory effectors, establishing a feed-forward loop that serves to protect the host.

Figure S1

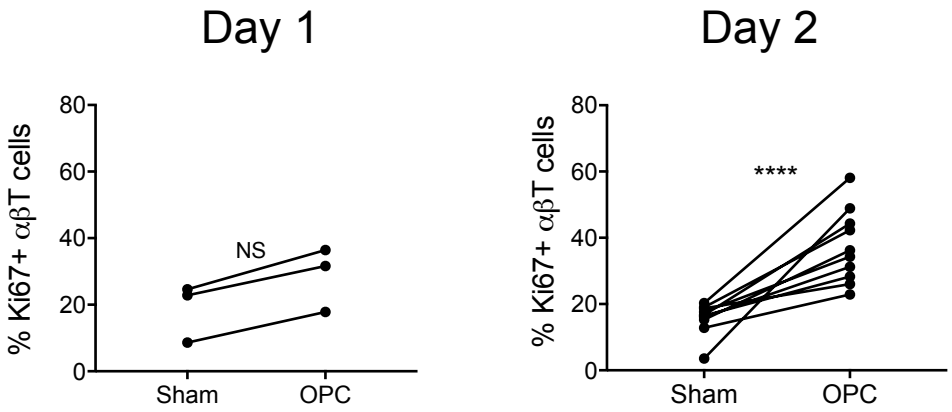


Figure S2

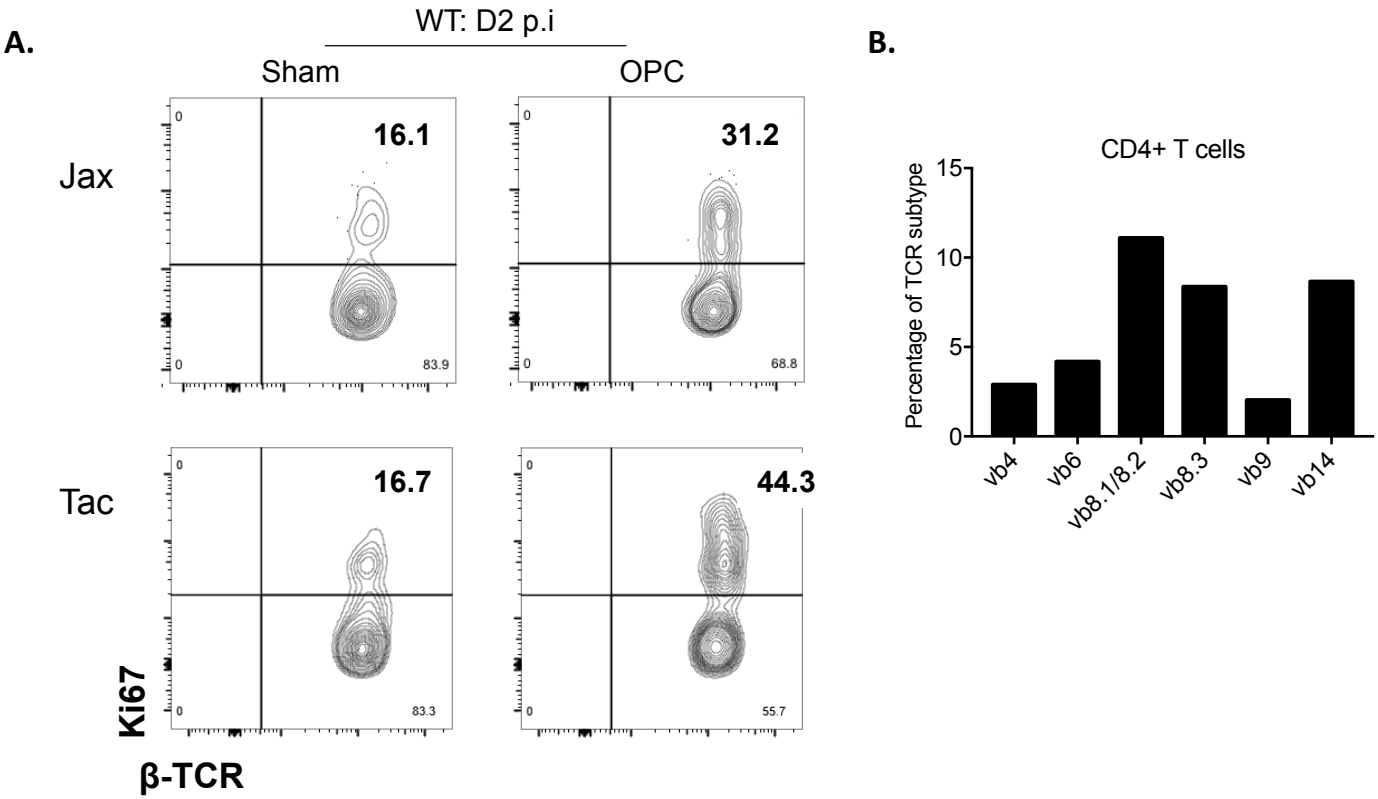

A.
WT: D2 p.i
Sham
OPC
Jax
Tac
16.1
31.2
16.7
44.3
83.9
68.8
83.3
55.7
Ki67
β-TCR
B.
CD4+ T cells
Percentage of TCR subtype
15
10
5
0
vb4
vb6
vb8.1/8.2
vb8.3
vb9
vb14

Figure S3

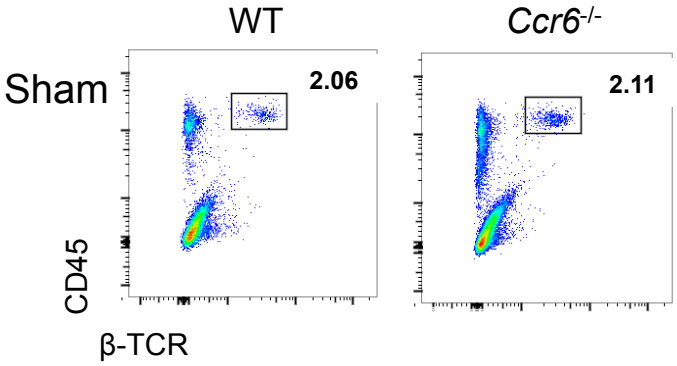


Figure S4

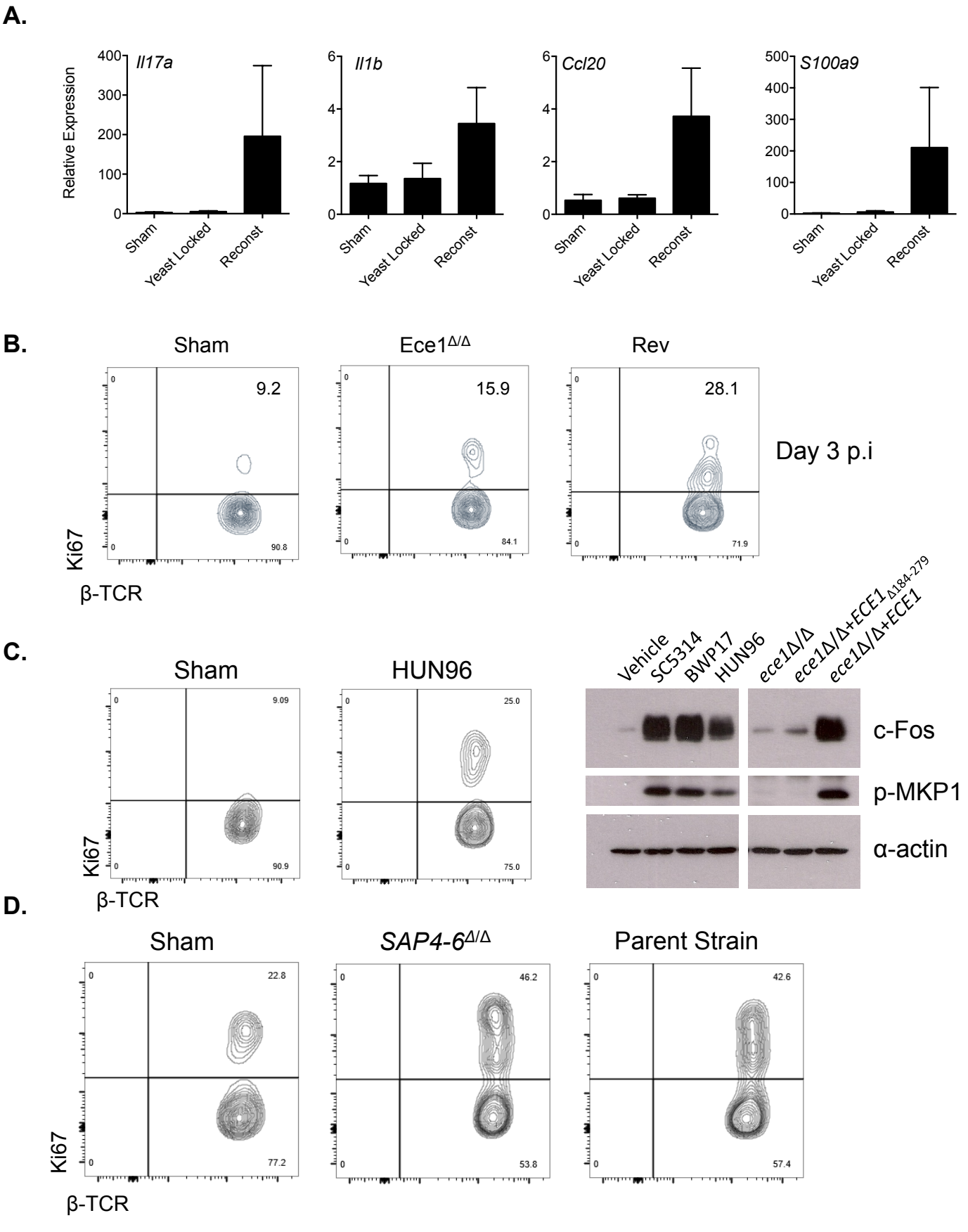

A.
Il17a
Il1b
Ccl20
S100a9
Relative Expression
Sham
Yeast Locked
Reconst
B.
Sham
Ece1Δ/Δ
Rev
9.2
15.9
28.1
90.8
84.1
71.9
Day 3 p.i
Ki67
β-TCR
C.
Sham
HUN96
9.09
25.0
90.9
75.0
Vehicle
SC5314
BWP17
HUN96
ece1Δ/Δ
ece1Δ/Δ+ECE1Δ184-279
ece1Δ/Δ+ECE1
c-Fos
p-MKP1
α-actin
D.
Sham
SAP4-6Δ/Δ
Parent Strain
22.8
46.2
42.6
77.2
53.8
57.4

Figure S5

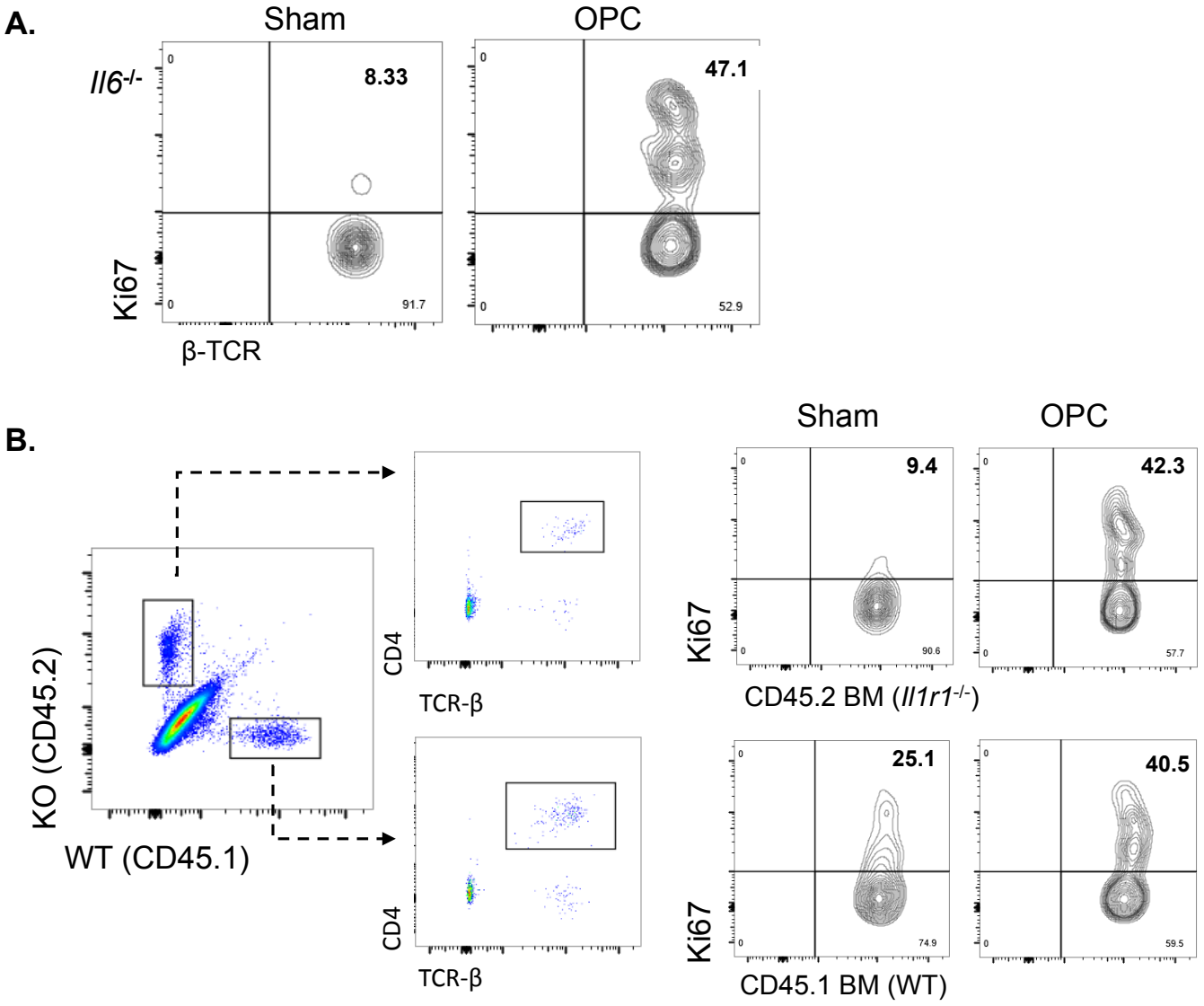

A.
Sham
OPC
Il6-/-
8.33
47.1
91.7
52.9
Ki67
β-TCR
B.
KO (CD45.2)
WT (CD45.1)
CD4
TCR-β
CD4
TCR-β
Sham
OPC
9.4
42.3
90.6
57.7
Ki67
CD45.2 BM (Il1r1-/-)
25.1
40.5
74.9
59.5
Ki67
CD45.1 BM (WT)
C.
CD4Cre Il1r1fl/fl → WT
Sham
OPC
13.1
33.3
86.9
66.7
Ki67
CD45.2 (CD4Cre Il1r1fl/fl)

Figure S6

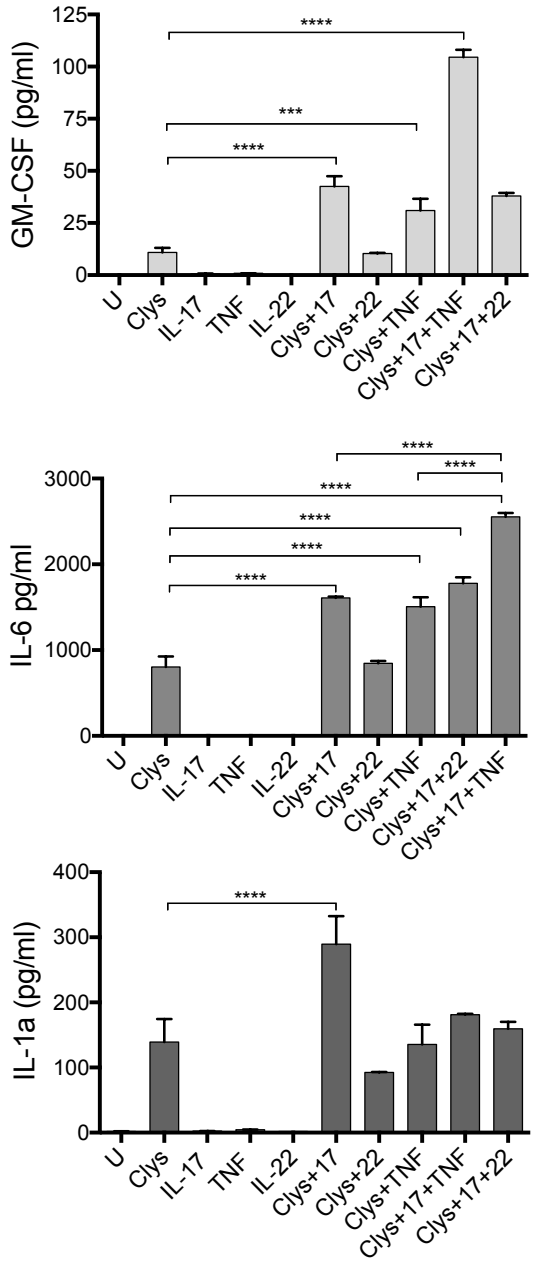
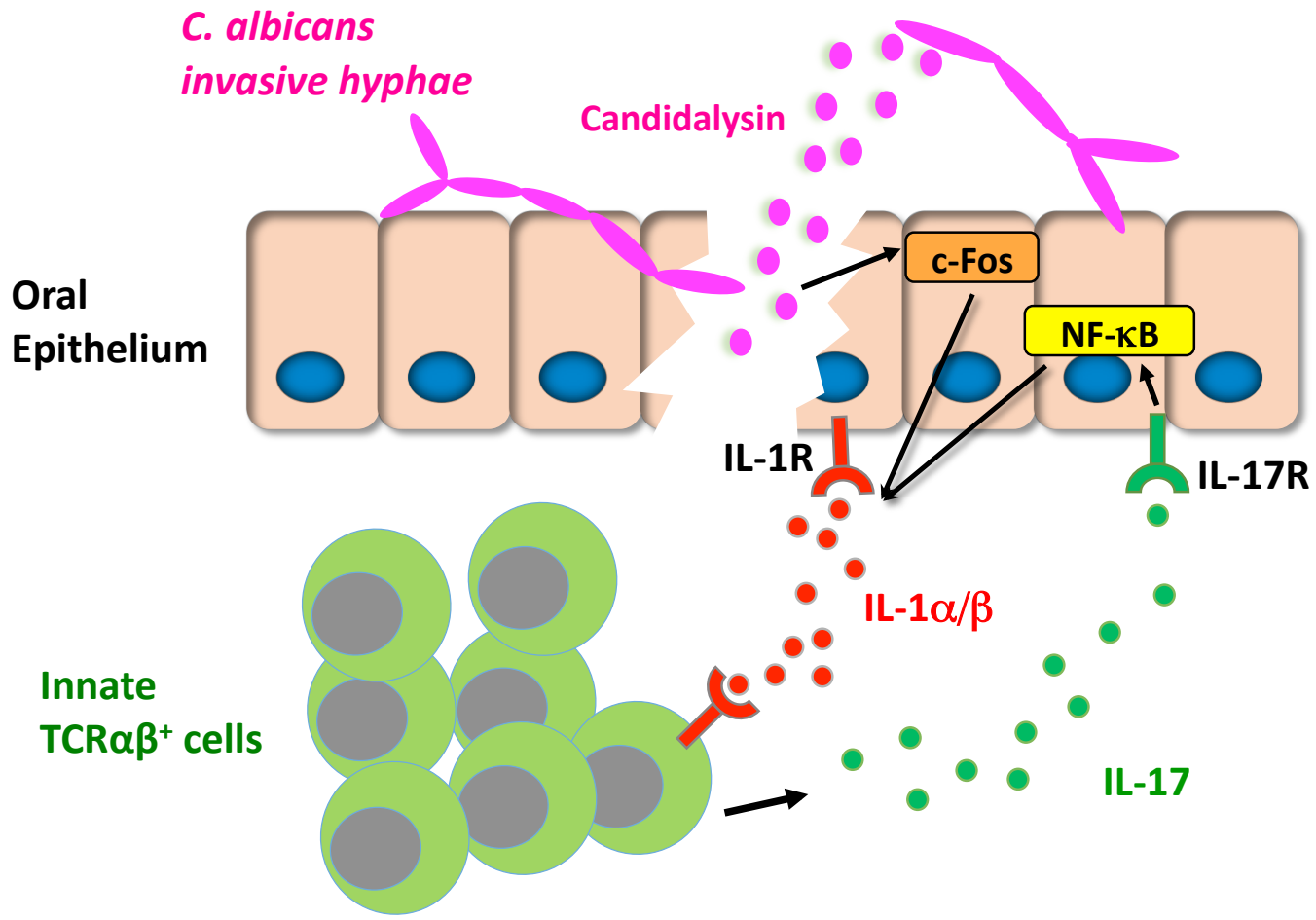

GM-CSF (pg/ml)
125
100
75
50
25
0
****
***
****
U
Clys
IL-17
TNF
IL-22
Clys+17
Clys+22
Clys+TNF
Clys+17+TNF
Clys+17+22
IL-6 pg/ml
3000
2000
1000
0
****
****
****
****
****
U
Clys
IL-17
TNF
IL-22
Clys+17
Clys+22
Clys+TNF
Clys+17+22
Clys+17+TNF
IL-1a (pg/ml)
400
300
200
100
0
****
U
Clys
IL-17
TNF
IL-22
Clys+17
Clys+22
Clys+TNF
Clys+17+TNF
Clys+17+22

Figure S7


C. albicans
invasive hyphae
Candidalysin
Oral
Epithelium
c-Fos
NF-κB
IL-1R
IL-17R
IL-1α/β
Innate
TCRαβ+ cells
IL-17