Oral-resident natural Th17 cells and γδ T cells control opportunistic *Candida albicans* infections

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Oropharyngeal candidiasis (OPC) is an opportunistic fungal infection caused by *Candida albicans*. OPC is frequent in HIV/AIDS, implicating adaptive immunity. Mice are naive to *Candida*, yet IL-17 is induced within 24 h of infection, and susceptibility is strongly dependent on IL-17R signaling. We sought to identify the source of IL-17 during the early innate response to candidiasis. We show that innate responses to *Candida* require an intact TCR, as SCID, IL-7Rα$^{-/-}$, and Rag1$^{-/-}$ mice were susceptible to OPC, and blockade of TCR signaling by cyclosporine induced susceptibility. Using fate-tracking IL-17 reporter mice, we found that IL-17 is produced within 1–2 d by tongue-resident populations of γδ T cells and CD3^{+}CD4^{+}CD44^{hi}TCRβ^{+}CCR6^{+} natural Th17 (nTh17) cells, but not by TCR-deficient innate lymphoid cells (ILCs) or NK cells. These cells function redundantly, as TCR-β$^{-/-}$ and TCR-δ$^{-/-}$ mice were both resistant to OPC. Whereas γδ T cells were previously shown to produce IL-17 during dermal candidiasis and are known to mediate host defense at mucosal surfaces, nTh17 cells are poorly understood. The oral nTh17 population expanded rapidly after OPC, exhibited high TCR-β clonal diversity, and was absent in Rag1$^{-/-}$, IL-7Rα$^{-/-}$, and germ-free mice. These findings indicate that nTh17 and γδ T cells, but not ILCs, are key mucosal sentinels that control oral pathogens.

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Abbreviations used: CMC, chronic mucocutaneous candidiasis; CsA, cyclosporin A; IF, immunofluorescent; ILC, innate lymphoid cell; nTh17, natural Th17; OPC, oropharyngeal candidiasis.

Oropharyngeal candidiasis (OPC; thrush) is an opportunistic fungal infection caused by the commensal yeast *Candida albicans*. In humans, T cells with reactivity to *Candida* in the blood are predominantly of the Th17 subset (Acosta-Rodriguez et al., 2007), and OPC is strongly associated with HIV/AIDS. To date there are no clinically approved vaccines against fungi, although experimental vaccines against *Candida* and other fungal species require Th17 cells (Spellberg et al., 2006; Lin et al., 2009; Wüthrich et al., 2011). Mice lacking IL-23, either IL-17R subunit or the adaptor Act1, are susceptible to oral and dermal candidiasis (Farah et al., 2006; Conti et al., 2009; Ho et al., 2010; Kagami et al., 2010; Ferreira et al., 2014). In keeping with data in mice, several diseases in humans demonstrate a protective role for IL-17 in chronic mucocutaneous candidiasis (CMC; encompassing recurrent oral, dermal, and vaginal candidiasis; Huppler et al., 2012; Milner and Holland, 2013). For example, in APS-1 (autoimmune polyendocrinopathy syndrome 1), CMC is associated with neutralizing antibodies against Th17 cytokines (Browne and Holland, 2010; Puel et al., 2010).

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Defects in Th17 cell frequency due to *STAT3* mutations cause CMC in Hyper-IgE/Job's syndrome. CMC also occurs in individuals with Th17 impairments due to mutations in *STAT1*, *CARD9*, *DECTIN1*, *IL12B*, or *IL12RB1* (de Beaucoudrey et al., 2010; Liu et al., 2011; Milner and Holland, 2013; Ouederni et al., 2014). Direct evidence for IL-17 signaling comes from patients with mutations in the IL-17 pathway (*IL17RA*, *IL17F*, or *ACT1*) who experience CMC but surprisingly few other infections (Puel et al., 2011; Boisson et al., 2013).

Production of IL-17 is classically associated with $CD4^+$ Th17 effector cells. Th17 differentiation is regulated by IL-6, TGF-β, and IL-1β via STAT3 and RORγt, with IL-23 serving as a vital maintenance factor (McGeachy et al., 2009; Haines et al., 2013). Th17 cells express antigen-specific αβ-TCRs and are enriched at mucosal surfaces, which have been best studied in the GI tract. Additionally, considerable recent work has identified various innate cell types that also express IL-17, collectively called Type 17 cells. Type 17 subsets include certain iNKT and γδ T cells, as well as a heterogeneous innate lymphoid cell (ILC) population known as ILC3 that lacks the TCR (Sutton et al., 2012; Spits et al., 2013). Unlike inducible Th17 cells that require activation by specific antigen, Type 17 cells can be mobilized within hours or days. Type 17 and Th17 cells also differ in their developmental requirements and expression of certain cell surface markers (Takatori et al., 2009; Buonocore et al., 2010; Sutton et al., 2012; Spits et al., 2013). Innate Type 17 cells bear many similarities to conventional Th17 cells, including dependence on RORγt and IL-23. In some circumstances, they may be equally or even more important than Th17 cells (Cua and Tato, 2010; Spits et al., 2013).

Natural Th17 (nTh17) cells are another poorly understood Type 17 cell population. These cells develop in the thymus and express IL-23R, RORγt, CD4, CCR6, α4β1 (VLA4, CD49d/CD29), and TCR-αβ (Marks et al., 2009; Tanaka et al., 2009; Zúñiga et al., 2013). In a double transgenic system where mice express a high affinity TCR against a transgenic antigen, self-reactive nTh17 cells were shown to populate gut, lung, and liver (Marks et al., 2009). nTh17 cells are distinguishable from inducible Th17 cells by a STAT3- and IL-6–independent developmental program (Tanaka et al., 2009; Massot et al., 2014), constitutive expression of CD44, and an intrinsic capacity for immediate activation in naive hosts (Tanaka et al., 2009). To date, the function of nTh17 cells in hosts with a normal immune system is enigmatic (Marks et al., 2009; Zúñiga et al., 2013), although a new study indicates a potential role in a model of psoriasis (Massot et al., 2014).

The innate immune response to *Candida* is poorly understood, in part because humans are exposed to *C. albicans* early in life and mount strong adaptive Th17 responses. In contrast, *C. albicans* is not a commensal in rodents (Iliev et al., 2012), providing the opportunity to distinguish innate versus adaptive immune compartments. In mice, inducible Th17 cells are found only after a prolonged rechallenge with *C. albicans*, verifying that mice are immunologically naive to this microbe and, moreover, that cross-reactive Th17 cells are not generated against microbiota or food antigens (Bär et al., 2012; Hernández-Santos et al., 2013). Nonetheless, WT mice mount rapid immune defenses to OPC in an IL-17R–dependent manner (Conti et al., 2009), indicating that there is an important contribution of the innate response in controlling *C. albicans* through IL-17. A controversial study suggested that ILC3s are involved in protection against OPC (Gladiator et al., 2013), but that publication did not demonstrate IL-17 production by those cells. Moreover, $Rag1^{-/-}$ mice are enriched for ILCs yet are susceptible to OPC (Pandiyan et al., 2011; Hernández-Santos et al., 2013).

Here, we used an acute model of oral candidiasis to identify the immediate innate sources of IL-17. Mice that cannot rearrange antigen receptors, such as $Rag1^{-/-}$, SCID, and IL-7Rα$^{-/-}$ mice, were susceptible to OPC. Analysis of the oral mucosa using an IL-17 reporter system showed no evidence for IL-17 production by cells lacking a TCR. Rather, IL-17 was expressed rapidly after *Candida* exposure by γδ T cells and also by a population of tissue-resident TCR-β^+ cells that are phenotypically consistent with nTh17 cells. These nTh17 cells were absent in $Rag1^{-/-}$, IL-7Rα$^{-/-}$, and germ-free mice, expressed CCR6 and the α4β1 integrin, and were IL-23–dependent but IL-6–independent. This is the first description of nTh17 cells in the oral mucosa, and these data indicate that nTh17 cells are positioned as sentinels to prevent infection by oral pathogens.

RESULTS

Acute immunity to OPC requires a rearranged TCR

We previously demonstrated that IL-23 and IL-17R signaling are essential for immunity to OPC (Conti et al., 2009; Pandiyan et al., 2011). It was evident that IL-17 must be produced by an innate immune cell type, as mice are naive to *C. albicans* yet fungal clearance occurred within 3–4 d. Moreover, in kinetic studies of OPC, there are almost no $CD4^+$ IL-17–producing cells in the draining cervical LN from mice after a short-term challenge with *C. albicans*, whereas Th17 cells are abundant in mice subjected to a secondary rechallenge (Bär et al., 2012; Hernández-Santos et al., 2013; Bishu et al., 2014). To define the early innate response to OPC, we first confirmed that there is no baseline expression of IL-17 in the oral mucosa in sham-infected WT mice. As shown, 24 h after oral inoculation with *C. albicans*, *il17* mRNA was strongly induced in the tongue, and expression was maintained for 3 d (Fig. 1 A). Expression was undetectable by 5 d, corresponding to fungal clearance (Kamai et al., 2001; Conti et al., 2009). Not surprisingly, immunity to OPC was dependent on Act1, a key adaptor for IL-17R signaling, and also on RORγt, a transcription factor required for IL-17 gene expression (Fig. 1, B and C). Thus, the response to acute OPC is mediated by an innate IL-23– and RORγt-dependent cell type via IL-17R/Act1 signaling.

IL-$6^{-/-}$ mice lack conventional Th17 cells (Yang et al., 2007) yet were fully competent to clear *C. albicans* from the oral cavity, suggesting that conventional Th17 cells are dispensable

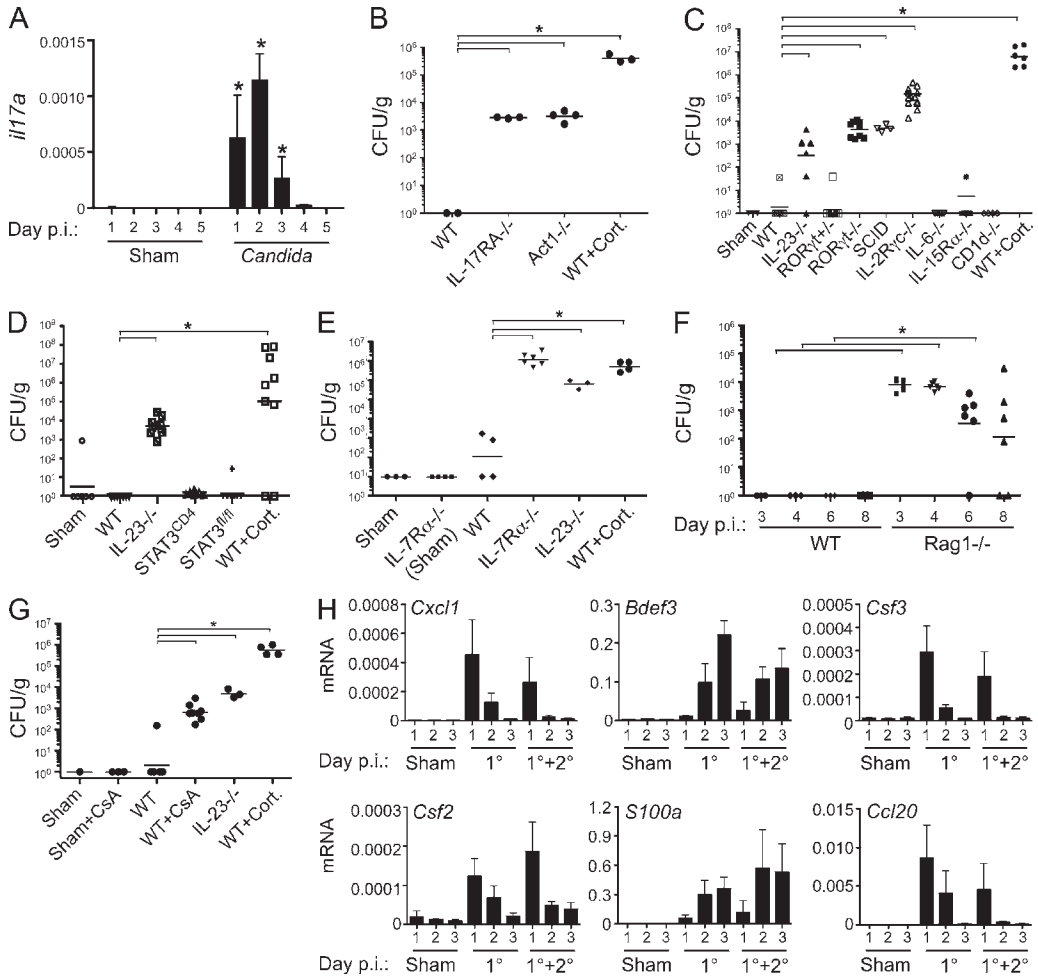

Figure 1. Innate immunity to OPC requires IL-17 signaling and a rearranged antigen receptor. (A) WT mice were subjected to OPC. At days 1–5, mRNA from pooled tongues ($n = 5$) was analyzed for *il17a* by qPCR in triplicate. *, $P < 0.05$ versus infected WT mice. (B–E) The indicated mice were subjected to OPC for 4–5 d, and fungal load was assessed by colony enumeration. Each point represents 1 mouse. Horizontal bars indicate geometric mean. WT mice were immunosuppressed with cortisone acetate (Cort.). *, $P < 0.05$ versus infected WT mice. Note: detection limit is 50 CFU/g. Each experiment was performed two or three independent times. (F) WT or Rag1$^{-/-}$ mice were subjected to OPC and fungal loads assessed at the indicated times. Horizontal bars indicate geometric mean. *, $P < 0.05$ versus infected WT mice. (G) The indicated WT mice were treated with CsA daily from day −2 and subjected to OPC. Fungal loads were assessed after 5 d. Horizontal bars indicate geometric mean. *, $P < 0.05$ versus infected WT mice. This experiment was performed twice. (H) Tongues were isolated from WT mice ($n = 5$) subjected to Sham inoculation, 5 d infection (1°), or 1° infection followed by rechallenge after 6 wk (1° + 2°). The indicated genes were evaluated in triplicate by qPCR. This experiment was performed three times. Error bars indicate SD.

for immunity to acute OPC (Fig. 1 C). Consistently, mice with a CD4-specific deletion of STAT3 (STAT3$^{fl/fl}$ x CD4-CRE, STAT3^{CD4}; Kaplan et al., 1996), which also lack conventional Th17 cells, were resistant to OPC (Fig. 1 D). IL-15Rα$^{-/-}$ mice, and CD1d$^{-/-}$ mice were also resistant to oral candidiasis, ruling out NK and NKT subsets. Moreover, strains that cannot rearrange the TCR, including SCID, IL-2Rγc$^{-/-}$, IL-7Rα$^{-/-}$, and Rag1$^{-/-}$ mice, were susceptible to OPC (Fig. 1, C–F). To determine whether signals through the TCR are necessary for immunity, cyclosporin A (CsA) was administered during infection. Mice treated with CsA exhibited fungal burdens similar to IL-23$^{-/-}$ or TCR-deficient mice, consistent with a requirement for intact TCR signaling (Fig. 1 G). Collectively, these data indicate that cells bearing a functional antigen receptor are necessary for innate immunity to OPC.

Notably, our data conflict with a report that Rag1$^{-/-}$ mice are resistant to OPC (Gladiator et al., 2013). In that paper, susceptibility was examined 7 d after infection, which is well past *Candida* clearance in WT mice. In an effort to reconcile these findings, we performed a kinetic analysis of OPC in Rag1$^{-/-}$ mice (Fig. 1 F). As shown, WT mice cleared *C. albicans* by day 3, whereas even 8 d after infection Rag1$^{-/-}$ mice reproducibly exhibited fungal colonization of the tongue. However, Rag1$^{-/-}$ mice started to show reduced fungal burdens by day 8, indicating that there may be protective cells starting to take effect at this later time point. Accordingly, in subsequent experiments we focused our attention on the early (1–2 d) IL-17–dependent response to OPC.

Innate IL-17–dependent responses to OPC resemble adaptive responses

To determine whether the innate IL-17 response to OPC was distinct in any way from a conventional Th17 response, we compared gene expression in tongue during a 1° and 2° *C. albicans* challenge. To generate Th17 cells against *Candida*, mice were subjected to a 1° infection followed by rechallenge 6 wk later, as previously described (Hernández-Santos et al., 2013; Bishu et al., 2014). We saw no difference in the pattern or magnitude of expression of a representative sample of known IL-17 target genes during 1° and 2° responses, including genes encoding chemokines (CXCL1), antimicrobial proteins (β-defensin 3), and cytokines (G-CSF; Conti et al., 2009; Fig. 1 H). This response independent of prior exposure supports the existence of an innate source of IL-17 in naive mice that is mobilized immediately upon *C. albicans* encounter.

Tissue-resident IL-17–expressing cells in the oral mucosa are TCR-γδ and natural Th17 cells

We next sought to characterize oral IL-17–expressing innate cells in detail. First, we used multiphoton confocal microscopy to visualize IL-17$^+$ cells in tongue of IL-17A–eGFP reporter mice. The papillae exhibited nonspecific autofluorescence (green), and collagen fluoresced in the red channel due to second harmonic generation (Fig. 2 A). GFP-positive IL-17–producing cells were not observed in Sham-infected mice but were present 2 d after infection (Fig. 2 A). Immunofluorescent (IF) staining revealed a small population of CD3$^+$ cells in Sham-treated mice. This population expanded considerably 2 d after infection (Fig. 2 B). To better define these cells, we developed a reliable protocol to phenotype viable cells from tongue by flow cytometry (Huppler et al., 2014). There was a small but highly reproducible population of CD45$^+$CD3$^+$TCR-β$^+$ cells in the tongue in Sham-infected mice (Fig. 2 C). As seen in the IF staining, this population expanded approximately twofold 48 h after *Candida* exposure (Fig. 2 C). There was also a substantial population of CD45$^+$CD3$^-$TCR-β$^-$ cells, which we have shown to consist mainly of neutrophils (Cd11b$^+$Gr1$^+$) recruited through IL-17–mediated induction of chemokines (Huppler et al., 2014).

The data in Fig. 2 suggested that IL-17–producing cells in tongue are not highly abundant. Therefore, we used a sensitive fate-tracking reporter mouse (Il17a^{Cre}Rosa26R^{eYFP}) that permanently marks IL-17–expressing cells with YFP (Hirota et al., 2011). In sham-infected Il17a^{Cre}Rosa26R^{eYFP} reporter mice, there was a small resident population of CD45$^+$ and CD44^{hi} cells in the tongue, which reproducibly expanded 2–3-fold after infection with *Candida* (Fig. 3 A). Importantly, only rarely did cells from sham-treated mice express YFP (reporting current or prior IL-17 expression), but a distinct population of cells was reproducibly YFP-positive 2 d after *Candida* exposure (Fig. 3, A and E).

In acute dermal candidiasis models, γδ T cells have been shown to be a major source of IL-17 (Kagami et al., 2010; Hirota et al., 2011; Igyártó et al., 2011). Like other mucosal sites, the tongue contains γδ T cells, primarily Vγ6 (Itohara et al., 1990; Prinz, 2011), although it has never been determined whether they express IL-17. In WT and Il17a^{Cre}Rosa26R^{eYFP} reporter mice, γδ T cells were present at a low frequency in tongue (mean of 0.2%; Fig. 3, B and D). Even before exposure to *Candida*, γδ T cells in the reporter mice showed evidence of present or prior IL-17 production (Fig. 3 E). After infection, there was a small increase in γδ T cell frequencies, although overall cell numbers remained low (Fig. 3 B, graph). We previously reported that TCR-δ$^{-/-}$ mice are largely resistant to OPC (Conti et al., 2009), which we reproduced here (Fig. 3 C). Thus, there are evidently compensatory mechanisms for protection against OPC in the absence of γδ T cells.

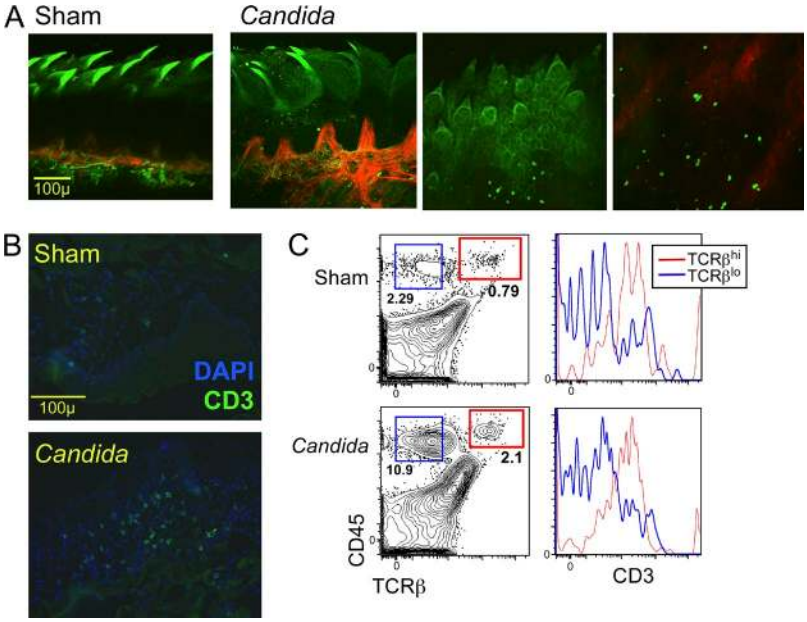

Figure 2. IL-17$^+$ and CD3$^+$ cells in the oral mucosa after *C. albicans* infection. (A) IL-17^{eGFP} reporter mice were subjected to OPC. 2 d later, tongue was subjected to 2-photon microscopy to visualize GFP. Note that papillae appear green due to endogenous autofluorescence. Bar, 100 µm. (B) Tongue sections from sham or *C. albicans*–infected RORγt$^{+/-}$ (surrogate for WT) mice were stained with α-CD3 or DAPI. Bar, 100 µm. (C) T cells in the tongue express CD3. Tongue homogenates from Sham- or *Candida*-infected WT mice were stained for CD45, TCR-β, and CD3. Red lines/boxes = TCR-β^{hi} cells; blue lines/boxes = TCR-β^{lo} cells. All experiments were performed twice.

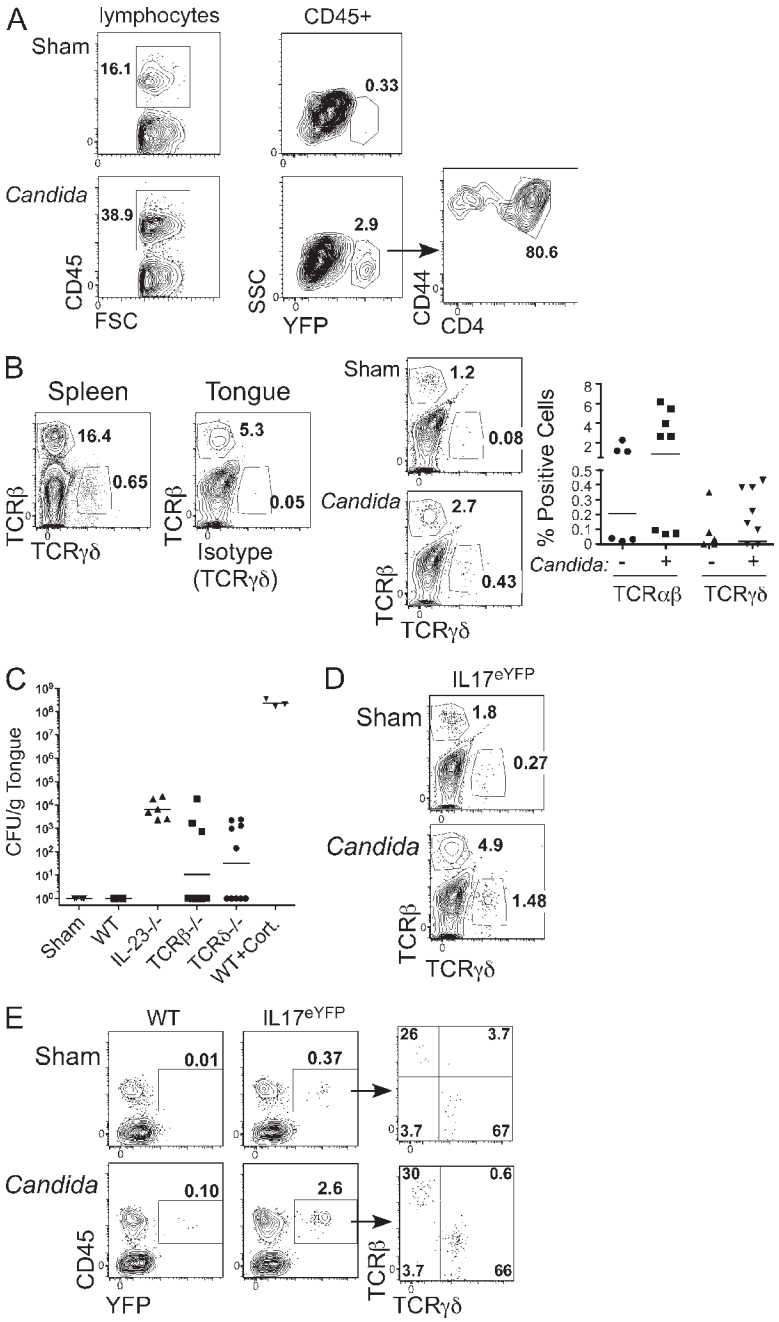

Figure 3. IL-17 is produced in the oral mucosa by γδT and nTh17 cells. (A) IL17^{Cre}Rosa26R^{eYFP} mice were subjected to OPC. 48 h later, cell homogenates from pooled tongues (n = 5) were stained for the indicated markers. Shown are CD45$^+$ populations in lymphocyte gate (left), and CD44$^+$ and CD4$^+$ populations or SSC and YFP in the CD45$^+$ gate (middle). In *C. albicans*–infected mice, the YFP$^+$ population was further gated on CD44 and CD4 (offset, far right). Data are representative of three independent experiments. (B) Cells from WT spleen (n = 1) or pooled tongues (n = 5) were stained for TCR-β and TCR-γδ (or isotype). The percentage of TCR-β$^+$ and TCR-γδ$^+$ cells (as a fraction of the total lymphocyte population) is indicated in each plot. The experiment was repeated 3 times, and pooled in graph at right. Horizontal bars indicate geometric mean. (C) TCR-β$^{-/-}$ and TCR-δ$^{-/-}$ mice were subjected to OPC and analyzed as in Fig. 1. Data are pooled from two independent experiments. Horizontal bars indicate geometric mean. (D) IL17^{Cre}Rosa26R^{eYFP} (IL-17^{eYFP}) mice were subjected to Sham infection or to OPC. Pooled cells from tongue (n = 5) were stained for CD45 (not depicted), TCR-β, and TCR-γδ. Experiment is representative of four independent replicates. (E) WT or IL-17^{eYFP} mice were subjected to Sham infection or to OPC. Pooled cells from tongue (n = 5) were stained for CD45, TCR-β, and TCR-γδ and visualized for YFP (two left panels). YFP$^+$ cells were analyzed for expression of TCR-β and TCR-γδ cells (right panel). The experiment is representative of two independent experiments.

In keeping with this idea, before *C. albicans* infection we observed a baseline population of TCR-β$^+$YFP$^+$ cells in the tongue (Fig. 3, D and E), which expanded after infection (Fig. 2 C; and Fig. 3, B, D, and E). These cells expressed CD4, distinguishing them from Type 3 ILCs or enthesis-resident pathogenic CD4$^-$CD8$^-$IL-17$^+$ cells (Fig. 3 A; Sherlock et al., 2012; Spits et al., 2013). This tongue-resident TCR-β$^+$ population was preserved in TCR-δ$^{-/-}$ mice (not depicted), consistent with the fact that TCR-δ$^{-/-}$ mice are resistant to OPC (Fig. 3 C). We applied high throughput sequencing technology (Immunoseq, Adaptive Biotechnologies) to assess clonal diversity in the oral nTh17 cell TCR-β repertoire (Robins, 2013). Sequencing indicated a highly diverse T cell population with a low clonality index, consistent with a broadly reactive population that has not undergone clonal expansion (unpublished data). Thus, *Candida*-induced expression of IL-17 in the oral mucosa derives not only from γδ$^+$ T cells but also from TCR-β$^+$ natural Th17 cells. Additionally, because TCR-β$^{-/-}$ mice are resistant to OPC (Fig. 3 C), it is likely that γδ T cells and nTh17 cells can functionally compensate for one another.

Phenotypic and functional characteristics of oral nTh17 cells

We next assessed the phenotypic characteristics of oral nTh17 cells. As expected, nTh17 cells were absent in tongues of

C. albicans–infected Rag1$^{-/-}$ and IL-7Rα$^{-/-}$ mice (Fig. 4, A and B). Interestingly, TCR-β^{+} cells were absent in germ-free mice, suggesting that commensal flora are needed for their development or recruitment to the oral mucosa (Fig. 4 C). To assess functional specificity, we infected mice orally with *Candida glabrata*, a distantly related fungal species that does not cause OPC in mice (Butler et al., 2009). There was no expansion of nTh17 cells upon exposure to *C. glabrata* (Fig. 4 D). It was recently suggested that the transcription factor PLZF may be a marker for nTh17 cells (Massot et al., 2014); although we saw no increase in PLZF expression in tongue after *C. albicans* infection, there were substantial baseline levels even before infection (Fig. 4 E). Consistent with nTh17 cells in other systems, the CD45^{+}TCRβ^{+} population expressed CCR6 and α4β1/VLA4 integrin (CD29 and CD49d) but did not strongly stain for CD49a (which is expressed on Th1 cells) or the α4β7/LPAM integrin (Fig. 4, F and G). The nTh17 cells were only weakly positive for Dectin-1 (Fig. 4 G), a PRR which recognizes fungal β-glucans (Brown and Netea, 2012). This is consistent with our observation that CARD9, a signaling adaptor downstream of Dectin family members, is dispensable for acute immunity to OPC (Bishu et al., 2014).

DISCUSSION

It has become appreciated that IL-17, and indeed most cytokines associated with effector Th cell subsets, are produced by parallel populations of specialized innate cells (Sutton et al., 2012; Spits et al., 2013). In some settings these may be more relevant than conventional Th17 cells, particularly at early time points after infectious challenge (Cua and Tato, 2010). We previously showed that the IL-17R is essential for immunity to OPC (Conti et al., 2009; Ho et al., 2010; Pandiyan et al., 2011), which has been verified in humans (Puel et al., 2011). However, the spectrum of cellular sources of IL-17 remained unclear. It was long assumed that conventional CD4^{+}Th17 cells are responsible for immunity to oral thrush because of its high incidence in HIV/AIDS and the observation that Th17 responses are generated in adults in response to *Candida* antigens (Acosta-Rodriguez et al., 2007; Fidel, 2011). However, the rapid time frame of fungal clearance in the OPC mouse model suggested that immunity to *C. albicans* in this system might be primarily innate (Kamai et al., 2001; Conti et al., 2009; Fig. 1). Supporting this idea is the observation that there are very few CD4^{+} IL-17^{+} cells in LN of mice that received a

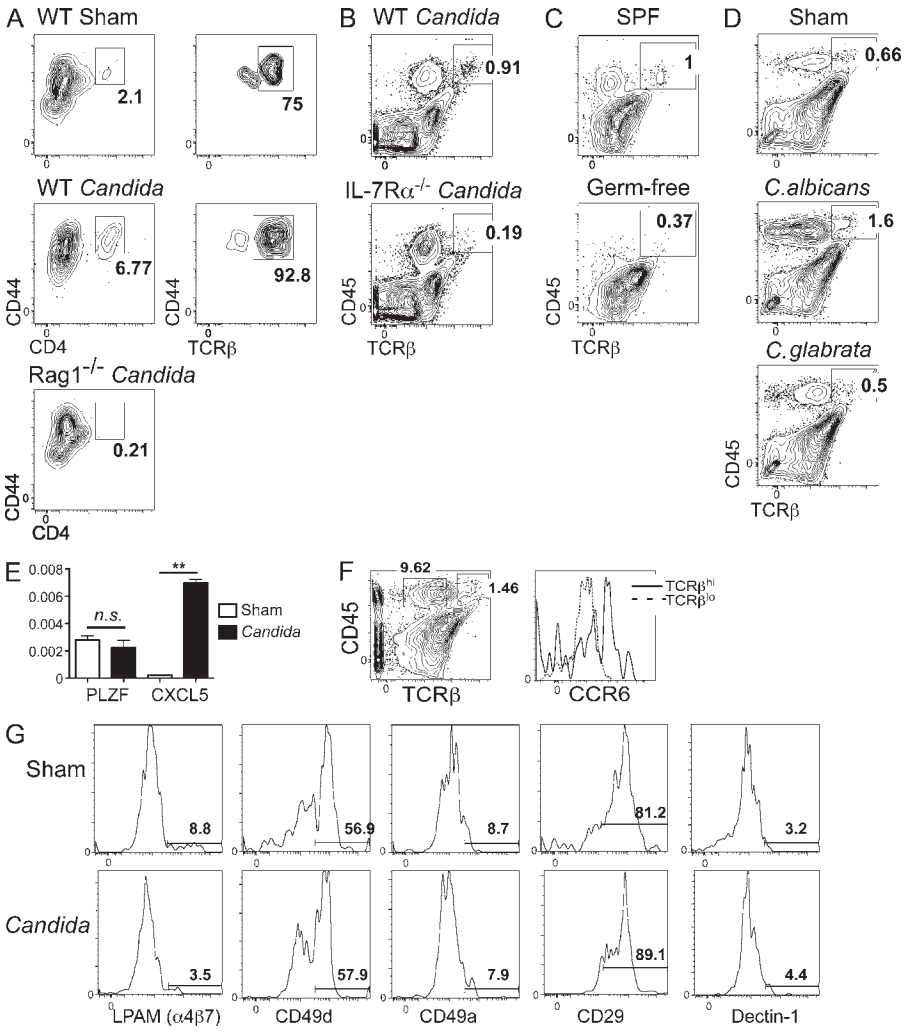

Figure 4. Phenotypic and functional characteristics of oral nTh17 cells. (A) WT or Rag1$^{-/-}$ mice were subjected to Sham inoculation or infected with *C. albicans*, and tongue homogenates were stained for the indicated markers. CD45^{+} cells were gated on CD4/CD44. Percentage of TCR-β^{+} cells is indicated. The experiment was performed twice. (B) WT or IL-7Rα$^{-/-}$ mice were infected with *C. albicans*, and tongue was stained for CD45 and TCR-β. The experiment was performed twice. (C) Tongue homogenates from mice housed in specific pathogen-free (SPF) or germ-free conditions was stained for CD45 and TCR-β. The experiment was performed twice. (D) WT mice were infected with *C. albicans* or *C. glabrata*, and tongue homogenates were stained for CD45 and TCR-β. Experiment was performed twice. (E) WT mice were subjected to OPC and 5 d later, mRNA was analyzed for expression of PLZF or CXCL5 as a control. The experiment was performed once. **, $P < 0.01$. Error bars indicate SD. (F) Tongue homogenates from WT *Candida*-infected mice were stained for CD45 and TCR-β and gated on CCR6. Solid = TCR-β^{hi}, dashed = TCR-β^{lo}. Experiment was performed twice. (G) Tongue homogenates from Sham- or *Candida*-infected WT mice were stained for the indicated markers and gated on lymphocytes. The experiment was performed twice.

primary challenge with *C. albicans*, whereas Th17 cells are abundant in mice subjected to a secondary challenge (Bär et al., 2012; Hernández-Santos et al., 2013). Here, we show that nTh17 cells play an unexpected role in innate protection against OPC. We also show for the first time that γδ T cells in the oral mucosa produce IL-17 in response to fungal infection.

γδ T cells are enriched at mucosal tissues (Martin et al., 2009; Ribot et al., 2009; Sutton et al., 2012). Although best studied in the GI tract and skin, they have been observed in the oral mucosa, albeit at low frequencies (Itohara et al., 1990; Prinz, 2011). In dermal candidiasis, γδ T cells are the main source of IL-17 (Kagami et al., 2010; Hirota et al., 2011; Igyártó et al., 2011), and i.p. injection of *Candida* or β-glucans stimulates recruitment of IL-17$^+$ γδ T cells (Martin et al., 2009). Their role in OPC was unclear, however, because TCR-δ$^{-/-}$ mice are largely resistant to oral candidiasis (Fig. 3 C; Conti et al., 2009). In this report, we show that γδ T cells express IL-17 after *Candida* infection (Fig. 3 B). Notably, γδ T cells can produce large quantities of IL-17 on a per-cell basis (Martin et al., 2009; Sutton et al., 2009), and therefore are likely to be an important source of oral IL-17. To our knowledge, this is the first direct demonstration that oral γδ T cells express IL-17.

nTh17 cells, in contrast, have received comparatively little attention (Zúñiga et al., 2013). Like conventional Th17 cells, nTh17 cells are thymically derived and express markers common to other IL-17–producing cells such as IL-23R, CCR6, and RORγt (Marks et al., 2009; Sutton et al., 2012; Massot et al., 2014). Because IL-7Rα$^{-/-}$ mice are susceptible to OPC (Fig. 4 B), it is conceivable that IL-7 serves as a growth factor in the absence of antigen stimulation. Although IL-6 is dispensable for nTh17 development, IL-23 is required (Tanaka et al., 2009; Massot et al., 2014). Consistently, we found that IL-23 is essential whereas IL-6 is dispensable for innate *Candida* responses (Fig. 1 C). Both cytokines signal via STAT3, and STAT3-insufficient humans are prone to CMC (Holland et al., 2007). We found that STAT3$^{fl/fl}$ mice crossed to the CD4 promoter/enhancer/silencer-CRE cassette (Chen et al., 2006) are resistant to acute OPC (Fig. 1 D); however, in these animals CRE is not expressed until the double-negative stage of thymic development (Lee et al., 2001) and nTh17 cells, presumably, are still able to develop. Therefore, the requirement for STAT3 in nTh17 cell activation remains unproven, though likely based on the critical role of IL-23.

Surprisingly little is known about the role of nTh17 cells in infection (Zúñiga et al., 2013). IL-17$^+$CD4$^+$ cells are present in DO11.10 (OVA-specific TCR transgenic) mice, and these cells mediated enhanced neutrophilic infiltration to lung after OVA administration (Tanaka et al., 2009). Regulation of neutrophils is a major activity of IL-17 and is consistent with its role in regulating immunity to OPC (Huppler et al., 2014). Notably, nTh17 cells were not found in DO11.10Rag1$^{-/-}$ mice (Tanaka et al., 2009), paralleling our findings (Fig. 1 F). Our work is therefore the first demonstration of a protective role for nTh17 cells in the context of a normal immune repertoire.

The capacity of both nTh17 cells and γδ T cells to express IL-17 helps explain the observation that TCR-β$^{-/-}$ and TCR-γδ$^{-/-}$ mice are both resistant to OPC (Fig. 3 C; Conti et al., 2009). A recent publication claims that IL-17–expressing ILCs protect against OPC (Gladiator et al., 2013). However, that report did not show directly that oral ILCs express IL-17, and the authors' conclusions were based on resistance of Rag1$^{-/-}$ mice at 7 d after infection, a very late time point. These results may be due to their selection of a high cutoff (>200 CFU/g), below which fungal loads were considered negative. We use a sensitive tissue processing and dilution method that allows detection of fungal loads as low as 50 CFU/g in a statistically robust manner, and in our hands Rag1$^{-/-}$ mice are susceptible to OPC. This is especially evident at early time points (days 1–3; Fig. 1 E). Nonetheless, by day 8 fungal loads in Rag1$^{-/-}$ mice appear to be decreasing, perhaps indicating the emergence of a later-acting antifungal cell type.

If a major source of IL-17 is from innate cells, why do humans mount such a vigorous conventional Th17 response to *C. albicans*? It is likely that the mouse OPC model best represents a first encounter to the fungus, akin to exposure in newborns, who are highly prone to thrush. Alternatively, the innate antifungal response may dominate over the adaptive response in rodents, perhaps explaining why *C. albicans* is not a commensal microbe in mice. In an early OPC study, CD4$^+$ T cells were found to be important for host immunity (Farah et al., 2002), although that work was performed before the recognition of Th17 cells and so IL-17 was not evaluated. In a 6-wk recall model, we observed a strong Th17 response to *C. albicans* in mice, which supplemented the innate response and reduced fungal loads by approximately one log (Hernández-Santos et al., 2013; Bishu et al., 2014). The present finding that nTh17 cells mediate the innate response to oral candidiasis was unexpected but may help explain the exquisite susceptibility of AIDS patients to oral thrush, because depletion of CD4$^+$ cells by HIV would presumably affect both conventional and nTh17 cells.

These studies reveal for the first time the involvement of nTh17 and γδ T cells as essential and immediate mediators of protection against oral mucosal *C. albicans*. This is also the first demonstration that nTh17 cells promote immunity to any infection, and it illustrates the vital role of innate immunity in promoting host defense against fungi.

MATERIALS AND METHODS

Mice. IL-23$^{-/-}$ mice were from Genentech and IL-17RA$^{-/-}$ mice from Amgen. IL-17A–eGFP reporter mice were from Biocytogen. IL-17^{CRE} mice, created by B. Stockinger (Hirota et al., 2011; The Jackson Laboratory) and crossed to Rosa26^{eYFP}. STAT3$^{fl/fl}$ and STAT3^{CD4} mice, were provided by Dr. J. O'Shea (National Institutes of Health). Tongues from germ-free mice and corresponding SPF controls were provided by the University of Pennsylvania Gnotobiotic Mouse Facility. Other mice were from The Jackson Laboratory. All mice were on the C57BL/6 background unless noted, and all experiments included appropriate age- and sex-matched controls.

Oral candidiasis model. OPC was performed by sublingual inoculation with a preweighed cotton ball saturated in *C. albicans* (strain CAF2-1) or *C. glabrata* (strain 74/042) for 75 min under anesthesia, as previously described (Kamai et al., 2001; Conti et al., 2009). For the rechallenge model, mice were reinfected 6 wk after a standard primary infection (Hernández-Santos et al., 2013). Oral swabs were obtained before every experiment to verify the

absence of commensal fungi. Controls were immunosuppressed with cortisone (225 mg/kg i.p.) on days −1, 1, and 3. Fungal loads in tongue were determined by dissociation on a GentleMACS (Miltenyi Biotec), followed by plating serial dilutions on YPD with antibiotics. 50 mg/kg CsA (Sandimmune; Novartis) i.p. was given daily from day −2 until sacrifice. Animal protocols were approved by the University of Pittsburgh IACUC. Data were analyzed on Excel and Prism (GraphPad Software), using ANOVA with post-hoc Tukey's test, Student's *t* test with Mann-Whitney correction, or Fisher's exact test.

Real-time PCR. Real-time quantitative PCR was performed on tongue as described, normalized to GAPDH (Hernández-Santos et al., 2013). Primers were from SuperArray (QIAGEN).

Confocal microscopy and flow cytometry. Whole tongue in PBS was visualized on a multiphoton confocal inverted microscope (Fluoview MPE; Olympus) at the Pittsburgh Center for Biological Imaging. For IF imaging, tongue was harvested on day 2, fixed in 4% paraformaldehyde, and embedded in OCT. 2 μM sections on glass were stained with DAPI and α-CD3 and Cy-3 Abs. Images were collected on a Provis instrument (Olympus). Flow cytometry of tongue tissue was performed as previously described (Huppler et al., 2014); in brief, pooled tongues (5 per sample) were processed with an enzyme cocktail (EDTA, collagenase-2 [Worthington Biochemical Corporation], dispase [Invitrogen], DNase I [Applied Biochemical], and defined trypsin inhibitor [Invitrogen]) or a Tumor Dissociation kit (Miltenyi Biotec) and incubated at 37°C for 45 min. Tissue was mechanically homogenized and passed through a cell strainer to form a single-cell suspension. Viability was >80% determined by live-dead staining, and the lymphocyte gate was verified by CD45 staining. The following Abs were from eBioscience, BD, or BioLegend: CD45–Alexa Fluor 700 (30-F11), CD44-e450 (IM7), CD4-FITC (GK1.5), TCR-β–PE (H57-597), and TCR-γδ–APC (GL3). Flow cytometry was performed on an LSRII or LSR Fortessa (BD) and analyzed with FlowJo (Tree Star).

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