Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis

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The commensal fungus *Candida albicans* causes oropharyngeal candidiasis (OPC; thrush) in settings of immunodeficiency. Although disseminated, vaginal, and oral candidiasis are all caused by *C. albicans* species, host defense against *C. albicans* varies by anatomical location. T helper 1 (Th1) cells have long been implicated in defense against candidiasis, whereas the role of Th17 cells remains controversial. IL-17 mediates inflammatory pathology in a gastric model of mucosal candidiasis, but is host protective in disseminated disease. Here, we directly compared Th1 and Th17 function in a model of OPC. Th17-deficient (IL-23p19$^{-/-}$) and IL-17R–deficient (IL-17RA$^{-/-}$) mice experienced severe OPC, whereas Th1-deficient (IL-12p35$^{-/-}$) mice showed low fungal burdens and no overt disease. Neutrophil recruitment was impaired in IL-23p19$^{-/-}$ and IL-17RA$^{-/-}$, but not IL-12$^{-/-}$, mice, and TCR-$\alpha\beta$ cells were more important than TCR-$\gamma\delta$ cells. Surprisingly, mice deficient in the Th17 cytokine IL-22 were only mildly susceptible to OPC, indicating that IL-17 rather than IL-22 is vital in defense against oral candidiasis. Gene profiling of oral mucosal tissue showed strong induction of Th17 signature genes, including CXC chemokines and β defensin-3. Saliva from Th17-deficient, but not Th1-deficient, mice exhibited reduced candidacidal activity. Thus, the Th17 lineage, acting largely through IL-17, confers the dominant response to oral candidiasis through neutrophils and antimicrobial factors.

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Abbreviations used: BD, β defensin; cLN, cervical LN; CM, conditioned media; HIES, hyper IgE syndrome; OPC, oropharyngeal candidiasis; PAS, periodic-acid Schiff.

Oropharyngeal candidiasis (OPC; "thrush") is an AIDS-defining illness caused mainly by the commensal fungus *Candida albicans* and related species (1). *C. albicans* also causes vaginal candidiasis, onychomycosis, and disseminated candidiasis. Disseminated candidiasis typically occurs as a nosocomial infection in neutropenic patients and has a 40% mortality rate (2). Interestingly, host defense mechanisms against *C. albicans* vary by anatomical site, as HIV-infected patients are primarily susceptible to oropharyngeal, but not vaginal or disseminated, candidiasis (1, 3, 4). Thus, CD4$^+$ T cells play a more central role in OPC compared with other forms of candidiasis, but the mechanisms by which this occurs remain poorly understood.

Recently, a new CD4$^+$ T effector cell was discovered that was distinct in lineage and cy-

tokine profile from classical Th1 and Th2 populations. Termed "Th17," these cells secrete IL-17 (IL-17A) and IL-17F, IL-21, and IL-22 (5). Whereas Th1 cells develop in response to IL-12 (a heterodimeric cytokine composed of IL-12p35 and IL-12p40 subunits), Th17 cells are induced to differentiate by a combination of TGF-β, IL-6, IL-1, and IL-21. The IL-12 family cytokine IL-23, comprised of IL-12p40 and the unique partner IL-23p19, is essential for Th17 cell expansion and function. Despite a common subunit, IL-12 and IL-23 have dichotomous functions in vivo (5, 6).

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Recent studies have revealed an essential role for IL-17 and other Th17 cytokines in host defense at mucosal surfaces such as lung and gut, mediated by induction of prototypical innate immune genes including neutrophil-activating factors (CXC chemokines, G-CSF, and ICAM-1), antimicrobial peptides (defensins, mucins, and S100 proteins), and acute phase proteins (IL-6, lipocalin 2, CRP, and SAA) (7–10). Consistent with a mucosal function, Th17 cells express the CCR6 and CCR4 receptors, which facilitate targeting to mucosal areas (11–13). The oral cavity has many features that distinguish it from other mucosal regions, such as saliva and unique microbiota (14). We recently showed that IL-17 plays a host-protective role in preventing periodontal bone loss after infection with a human periodontal pathogen through activation of neutrophils (15, 16). However, the role of IL-17 and Th17 cells in coordinating host defense in the oral mucosa is largely undefined.

Accumulating evidence implicates IL-17 and Th17 cells in host defense against fungi, but no prior studies have examined these cells in OPC. IL-17 receptor–deficient (IL-17RA^{KO}) mice are susceptible to disseminated *C. albicans* through regulation of neutrophils, particularly via CXC chemokines (17). The pattern recognition receptor dectin-1 binds to β-glucan components of yeast cell walls and was reported to drive a protective Th17 response in models of disseminated candidiasis (18, 19). In humans, resident memory T cells specific for *C. albicans* are primarily of the Th17 subset (11, 20). IL-17 also contributes to host defense against other fungi, including *Pneumocystis carinii* (21). Conversely, in mouse models of fungal gastric candidiasis or aspergillosis, IL-17 and IL-23 have been reported to play a counterproductive role by amplifying inflammatory pathology (22).

It has long been believed that immunity to OPC is mediated by both neutrophils and Th1 cells (2). Although neutrophils are classically considered part of the innate immune response, IL-17 is a critical regulator of neutrophils through CXC chemokines and granulopoietic cytokines (23). IL-12p40^{KO} mice are deficient in Th1 cells and are susceptible to OPC (24); however, IFN-γ^{KO} mice also have an impaired Th1 response and are resistant to oral candidiasis (24). Because IL-12p40 is a common component of IL-12 and IL-23, we hypothesized that a Th17-mediated response rather than a defective Th1 response is responsible for the increased susceptibility of the IL-12p40^{KO} mice to OPC.

To dissect host responses to OPC systematically, we used a model of OPC (25) using mice deficient in Th1 cells (IL-12p35^{KO}), Th17 cells (IL-23p19^{KO}), IL-17RA (the receptor for IL-17 and IL-17F), or IL-22. Here, we show that IL-17RA^{KO} and IL-23^{KO} mice are profoundly susceptible to oral candidiasis, whereas IL-12^{KO} mice show only a slight increase in fungal burden and no overt fungal lesions. Analyses of the oral mucosa revealed induction of IL-17 target genes encoding neutrophil-activating factors, CXC-chemokines, and antimicrobial proteins in WT mice after oral *C. albicans* infection. The majority of these genes were suppressed in IL-17RA^{KO} mice after infection, particularly β defensin 3 (BD3).

Saliva from IL-17RA^{KO} and IL-23^{KO} mice showed a reduced capacity to kill *C. albicans* in vitro, which correlated with reduced levels of BD3 in the oral mucosa. Thus, immunity to OPC appears to be dominantly regulated by IL-17 signaling rather than Th1 cells.

RESULTS

Th17–deficient and IL-17 receptor–deficient mice are susceptible to oral candidiasis

To dissect T cell responses during OPC, we used a quantitative mouse model of OPC (Fig. 1 A) in which mice were infected sublingually with 2×10^7 *C. albicans* blastospores/ml. The extent of candidiasis was evaluated 5 d later by tissue fungal burden (Fig. 1 B) and histology (Fig. 1, C–E). None of the mice exhibited detectable carriage of oral *C. albicans* based on an oral swab taken before the first infection (unpublished data). Similar to immunocompetent humans, WT mice infected with *C. albicans* are almost completely resistant to infection, with an extremely low fungal burden 5 d after inoculation (Fig. 1, B–D). Inoculation of *C. albicans* into immunocompetent WT mice was associated with a strong neutrophilic infiltration into the oral cavity, in the epithelial, basal, and muscular layers of the tongue (Fig. 1 D). When WT mice were immunosuppressed with cortisone acetate, a mean of 1.5×10^6 CFU/g (geometric mean of 6.4×10^5) was recovered from tongue ($n = 16$; Fig. 1 B; see also Fig. 2 A), which is consistent with prior findings (25). These mice lost considerable body weight (Fig. 1 F) and exhibited fungal patches covering the dorsal layer of the tongue, palate, and buccal mucosa (Fig. 1 G). Microscopic analysis indicated that yeast invaded the superficial epithelial layer of the mucosa, destroying the papillae and the overall architecture of the keratinized superficial epithelium. There was extensive proliferation of the basal germinal layer, which is indicative of ongoing tissue repair. Although some neutrophils and eosinophils were found at infected sites, this was substantially reduced compared with WT mice (Fig. 1 D vs. Fig. 1 E). Both hyphal and blastoconidial forms of *C. albicans* were present (Fig. 1 E). Thus, this model faithfully reproduces key clinical features of human OPC (25).

To compare Th1 and Th17 cells in OPC directly, IL-12p35– and IL-23p19–deficient mice (hereafter, IL-12^{KO} and IL-23^{KO}) were infected with *C. albicans*. IL-23^{KO} mice showed a fungal burden of 2.6×10^4 CFU/g, which was less than cortisone-treated WT mice, but nonetheless indicated considerable susceptibility to OPC (Fig. 2 A). In contrast, IL-12^{KO} mice (1.4×10^3 CFU/g) were ~450-fold less susceptible than cortisone-treated WT mice and ~20-fold less susceptible than IL-23^{KO} mice, indicating that IL-23 is far more important for mediating host defense against OPC than IL-12. Weight loss values were consistent with recovery of *C. albicans* cells from tongue, as IL-23^{KO} mice showed continuous weight loss, whereas IL-12^{KO} mice showed transient weight loss at day 2, but recovered by day 5 (Fig. 2 B). Although characteristic *C. albicans* lesions were readily visible on the palates and tongues of IL-23^{KO} mice, no fungal lesions

were found in the IL-12[KO] mice (Fig. 2 F). Hyphal formation and invasion of the superficial epithelial layer were visible in IL-23[KO] tongue sections, confirming invasive candidiasis (Fig. 2 D). Disruption of the superficial epithelium and some infiltration of the infected area with PMNs was observed. However, the degree of neutrophilic infiltrate was significantly reduced compared with WT or IL-12[KO] mice, and PMNs appeared to be clustered in "nests" rather than crossing into
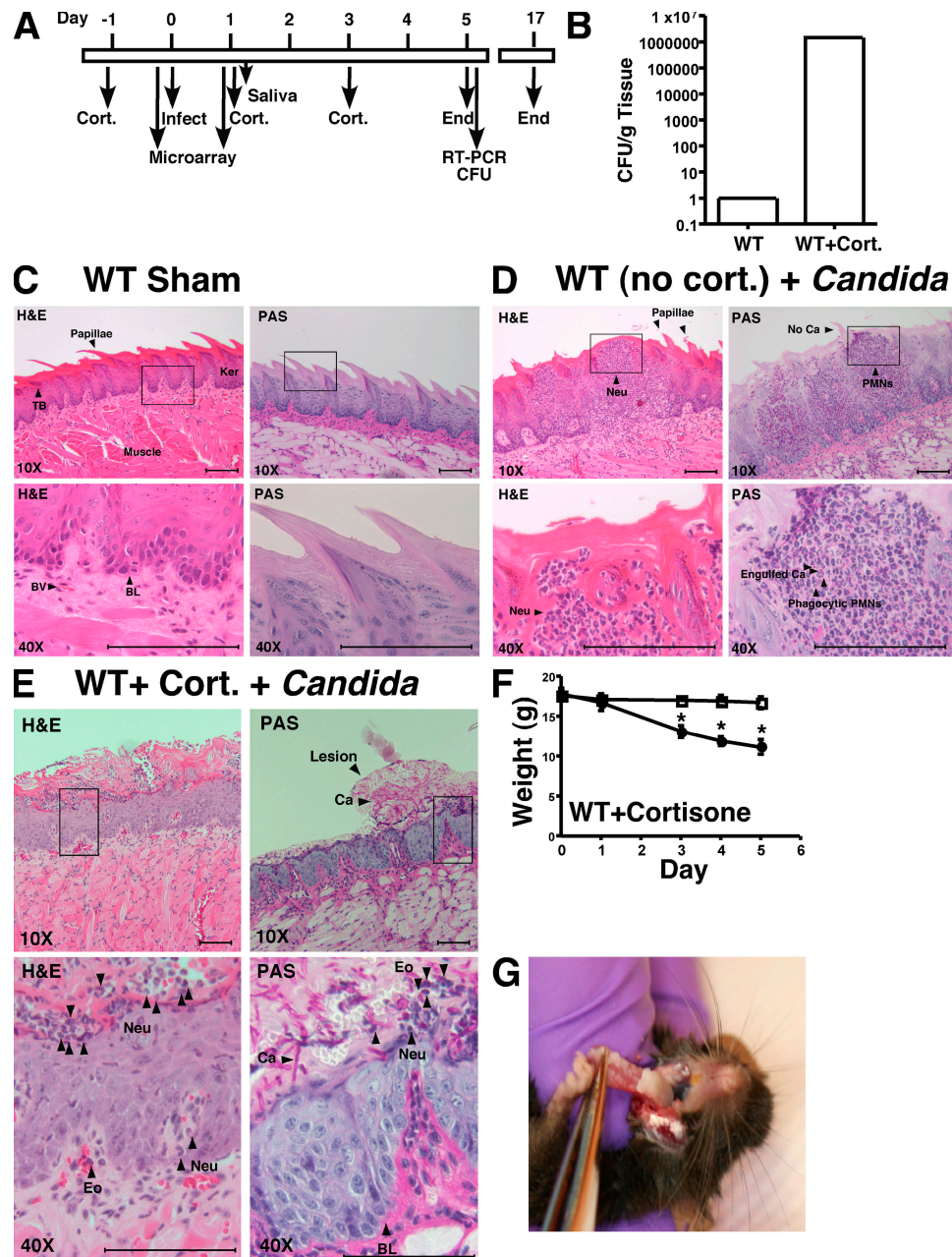

Figure 1. Model of murine OPC. (A) Timeline of infection model. (B) WT mice are resistant to OPC unless they are immunosuppressed. WT mice ($n = 8$) or WT mice treated with cortisone acetate ($n = 16$) were infected sublingually with *C. albicans*, and after 5 d CFU/g of tongue tissue was assessed in triplicate (log scale). These data are from at six independent experiments. (C–E) Histological evaluation of *C. albicans* infection. At day 5, sections of tongue from the indicated mice were stained with PAS to visualize *C. albicans* (Ca) or hematoxylin and eosin (H&E; which does not stain fungi) and viewed at 10X or 40X magnification. Fungal lesions, papillae, and keratinized layer are indicated. (C) WT mice subjected to sham infection. (D) WT mice without cortisone were infected orally with *C. albicans*. (E) WT mice treated with cortisone acetate were infected orally with *C. albicans*. TB, taste bud; BV, blood vessel; BL, basal layer; ker, keratinized layer; Neu, neutrophil; Eo, eosinophils; Ca, *C. albicans*. Data are representative of 2–3 mice per strain. Bars, 100 μm. (F) OPC induces weight loss in cortisone-treated WT mice. All mice were weighed daily, and weight (gram) on each day of infection is indicated. *, $P < 0.05$. Circles, Sham-infected mice; squares, *C. albicans*–infected mice. (G) OPC on day 5 after inoculation with *C. albicans*. Fungal lesions on tongue of WT mice treated with cortisone acetate.

the infected areas (Fig. 2, D and E). Fungal invasion in the IL-23^{KO} mice was less profound than in cortisone-treated mice (Fig. 1 E vs. Fig. 2 D). In contrast to IL-23^{KO} or cortisone-treated mice, there was little damage to the epithelial layer in the IL-12^{KO} mice, and *C. albicans* cells were almost never found on the surface or invading epithelial tissue (Fig. 2 C and not depicted). A prominent neutrophilic infiltrate occurred both in the muscular area and the subepithelial layer,
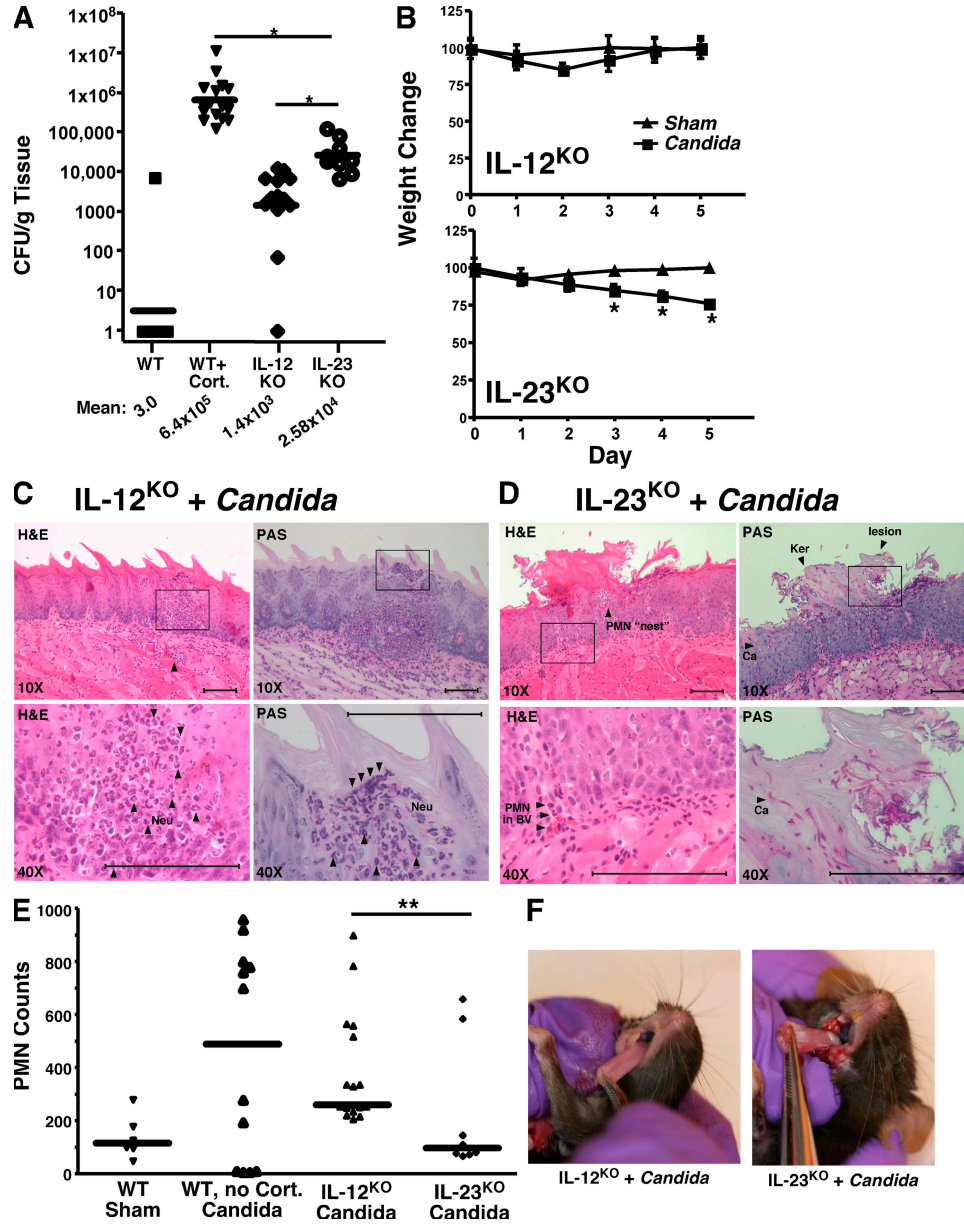

Figure 2.   Th17–deficient mice are more susceptible to OPC than Th1–deficient mice. (A) Quantitation of *C. albicans* infection in Th1-deficient (IL-12^{KO}) and Th17-deficient (IL-23^{KO}) mouse strains. IL-12^{KO} mice (n = 13) and IL-23^{KO} mice (n = 9) were infected orally with *C. albicans* and CFU/gram tongue tissue was assessed. Each dot represents an individual mouse, and horizontal bar indicates the geometric mean, which is also indicated below. *, P < 0.05 by Mann-Whitney *t* test. Note, the WT mouse data are the same as that shown in Fig. 1 B. IL-12^{KO} mice were evaluated in three independent experiments, and IL-23^{KO} mice were evaluated in two independent experiments. (B) OPC-induced weight loss in Th1-deficient or Th17-deficient strains. Mice were weighed daily and percentage of weight change compared with day 0 is shown for each strain. *, P < 0.05. Triangles, Sham-infected mice; squares, *C. albicans*–infected mice. (C and D) Histological evaluation of *C. albicans* infection in IL-12^{KO} versus IL-23^{KO} mice. At day 5, sections of tongue from the indicated mice were stained with PAS or hematoxylin and eosin (H&E) and viewed at 10–40X magnification. Data are representative of two mice per strain. Bars, 100 μm. (E) Quantitation of histological evaluation of PMNs. Six sections per mouse from two mice per group were evaluated for the number of PMNs per microscopic field, either in the vicinity of fungal lesions or parallel to areas of sham-infected tongue. Evaluators were blinded to the identity of the samples. (F) OPC on day 5 after inoculation with *C. albicans*. Fungal lesions on tongues of representative IL-12^{KO} or IL-23^{KO} mice are shown.

indicative of an ongoing immune response (Fig. 2, C and E). Thus, IL-23, but not IL-12, is required to prevent invasive oral candidiasis and permit a robust PMN response.

IL-23 drives expansion of the Th17 lineage, and IL-17 and IL-17F are hallmark Th17 cytokines that bind to a common receptor, IL-17RA. The oral fungal burden (2.64×10^4 CFU/g; Fig. 3 A) and weight loss (Fig. 3 B) in IL-17RA[KO] mice were not statistically different from the IL-23[KO] mice and were higher than the IL-12[KO] mice (compare Fig. 3 to Fig. 2; statistical comparisons not depicted). Fungal lesions were visible on the tongue and palate at levels similar to the IL-23[KO] mice (not depicted), and invading hyphae were frequently found in IL-17RA[KO] tongue sections (Fig. 3 D). Destruction of the epithelial and basal layers was observed in IL-17RA[KO] sections, similar to the IL-23[KO] mice. Some neutrophilic infiltrates were present, similar to the IL-23[KO] mice, but were less numerous than in IL-12[KO] or WT tissue (Fig. 3 D). In IL-17RA[KO] mice, as in IL-23[KO] mice, PMNs appeared to be trapped in "nests," and there was no evidence of *C. albicans* phagocytosis as there was in the WT mice (compare with Fig. 1 D). Thus, IL-17 receptor signaling appears to account for much if not all of the IL-23–mediated protection, which is apparently caused in part by a failure to recruit PMNs into infected tissue.

We next assessed IL-17 expression in T cells from the draining cervical LN (cLN) after infection. Cortisone acetate treatment results in reduced lymphocyte numbers, so it was not surprising that WT mice treated with corticosteroids had extremely small cLNs, and not enough viable lymphocytes could be recovered to assess cytokine expression (unpublished data). However, IL-17RA[KO] mice (which can produce IL-17, but not respond to it) showed significantly increased numbers of IL-17^{+} T cells after infection compared with uninfected mice (Fig. 3 C). Even without additional stimulation of the TCR, ~1% of the cLN lymphocytes stained positive for IL-17, comparable to reports with *Toxoplasma gondii* infection (26). After 2 d of in vitro restimulation of the TCR, ~12% of T cells expressed IL-17, compared with <1% in sham-treated mice. Thus, IL-17–producing cells are activated after oral *C. albicans* infection. Moreover, sham-treated mice do not harbor substantial numbers of IL-17–expressing T cells before infection.

Next, we questioned whether IL-17RA[KO] mice could eventually clear *C. albicans*, or whether IL-17 signaling was necessary to recover from OPC. Accordingly, IL-17RA[KO] mice were monitored for weight loss and signs of infection for 2.5 wk (Fig. 3 G). Whereas WT mice showed almost no fungal burden 5 d after inoculation and fully recovered their body weight (Fig. 1 F), IL-17RA[KO] mice lost weight (Fig. 3 H) and maintained a mean fungal burden of 6.8×10^5 CFU/g *C. albicans* throughout a 17-d time course. In contrast, IL-12[KO] mice showed an early spike in fungal burden in the tongue, but fungal clearance and weight recovery were evident by the end of the experiment (Fig. 3, G and H). These data indicate that, whereas the oral fungal burden of the IL-17RA[KO] mice was lower than WT mice treated with steroids

(which must be killed by day 5 for humane reasons), OPC cannot be resolved in the absence of IL-17RA signaling. In contrast, IL-12 and hence Th1 cells are not essential for host defense against oral candidiasis, although they do contribute to some extent to limiting infection.

In an attempt to determine whether IL-17 is sufficient to rescue the Th17 deficiency, we added IL-17 orally twice daily to IL-23[KO] mice throughout a 5-d course of infection (unpublished data). However, ectopic application of IL-17 led to a systemic reaction characterized by reddening of the skin in the face and chest, and IL-17–treated mice were noticeably sicker than controls. There was no statistically significant difference in oral fungal burden between the groups (unpublished data). Accordingly, the confounding systemic effects of IL-17 may have counteracted any beneficial effects in the oral cavity.

Th17 cells also produce IL-22, which has been shown to play a more vital role than IL-17 in host defense against certain gut and lung mucosal pathogens (27, 28). Therefore, we evaluated the effect of an IL-22-deficiency in OPC. However, IL-22[KO] mice showed only a mild susceptibility to OPC compared with IL-17RA[KO] mice, although they lost weight after infection (Fig. 3, A and B). Consistent with their ability to largely combat fungal infections, IL-22[KO] mice showed robust neutrophilic infiltrates into the infected lesion sites (Fig. 3 D). There was no evidence of overt fungal lesions on the tongue, either visually (not depicted) or histologically (Fig. 3 D). Therefore, in contrast to lung and gut, IL-17 signaling appears to play a more important role in mediating immunity to infection in the oral cavity than IL-22 (see Discussion).

αβ–TCR and CD8^{+} cells, and not γδ–TCR cells or IL-22, contribute to immunity to OPC

IL-17 is produced by CD4^{+} and CD8^{+} cells that express the αβ-TCR, and also by an innate population of γδ-TCR T cells in an IL-23–dependent manner (29). In *Escherichia coli*, *Mycobacterium tuberculosis*, and *Klebsiella pneumonia* settings, the γδ-T cell population appears to be the dominant IL-17–producing T cell subset involved in host defense (30, 31). To determine which subset was primarily responsible for immunity against OPC, αβ-T cell–deficient mice or γδ-T cell–deficient mice were infected with *C. albicans*. Low levels of infection were observed in γδ-TCR[KO] mice, whereas substantial infection occurred in αβ-TCR[KO] mice (Fig. 4 A). These data are consistent with the sensitivity of HIV^{+} individuals to OPC (3) and the finding that PBMCs from healthy human donors contain *C. albicans*–specific memory CD4^{+} Th17 cells (11, 20). CD8^{+} T cells and innate CD8^{+} NKT cells also secrete IL-17 (32, 33), and CD8[KO] mice showed a similar susceptibility to OPC as the αβ-TCR[KO] mice (Fig. 4 A). Interestingly, the fungal burden in αβ-TCR[KO] mice was reproducibly lower than the IL-17RA[KO] or IL-23[KO] mice (Fig. 4 A vs. Fig. 2 A and Fig. 3 A). Consistent with this observation, the αβ-TCR[KO] mice did not lose as much weight as IL-23[KO] or IL-17RA[KO] mice (Fig. 4 B vs. Fig. 2 B and Fig. 3 B).
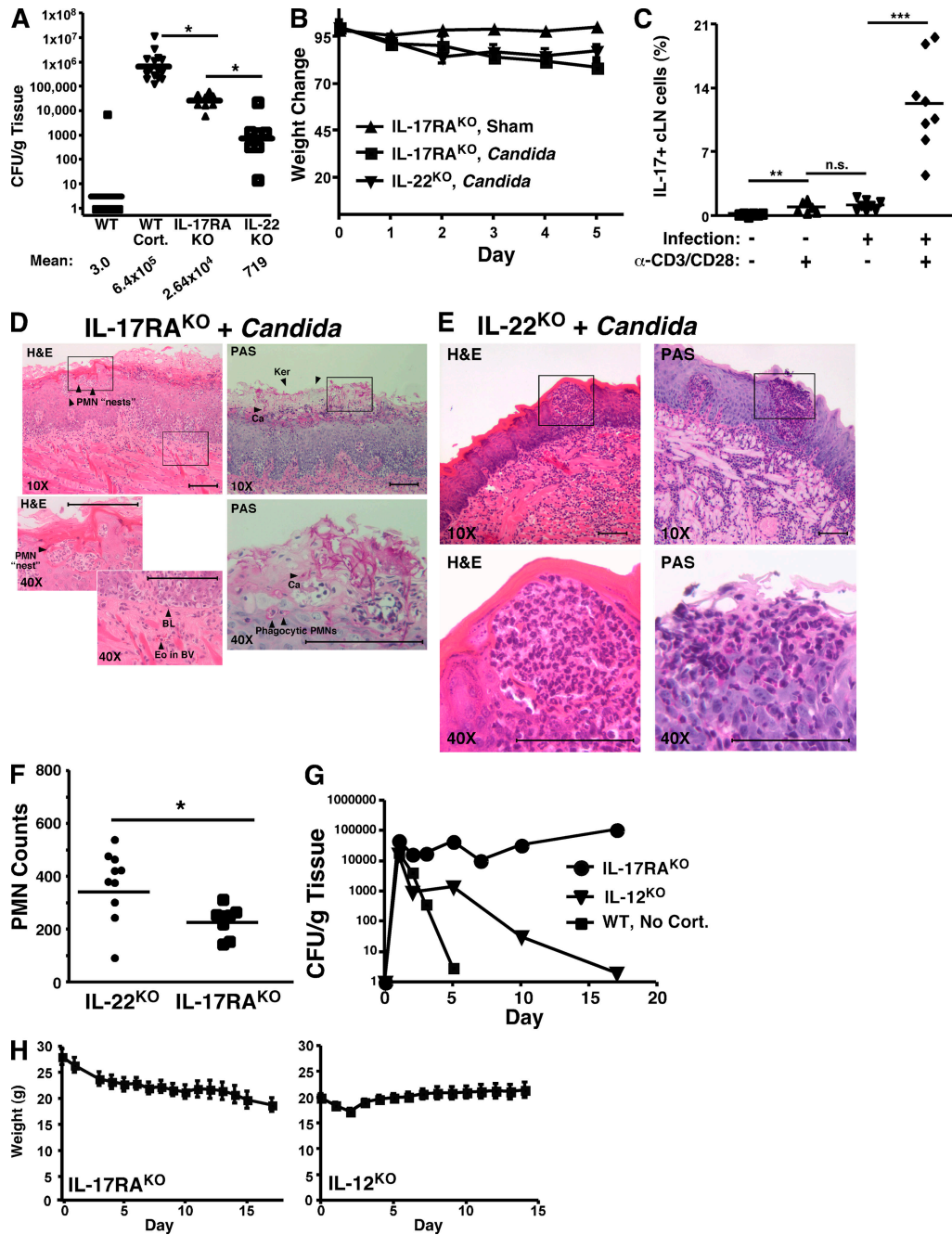
Figure 3. IL-17 signaling is essential for host defense against OPC. (A) IL-17RA[KO] mice are sensitive to OPC. The indicated mouse strains (IL-17RA[KO], *n* = 8; IL-22[KO], *n* = 7) were infected with *C. albicans* and CFU/g tongue was assessed by colony enumeration. *, P < 0.05. Geometric mean is indicated by horizontal bars and below. Note, data from WT mice are the same as shown in Fig. 1 B. IL-17RA[KO] mice data are representative of three independent experiments, and IL-22[KO] data were from one experiment. (B) OPC-induced weight loss in susceptible strains. Percentage of weight change compared with day 0 is shown. (C) IL-17^{+} cells are induced after *C. albicans* infection. Cells were isolated from the draining cervical lymph nodes of infected IL-17RA[KO] mice. Cells were incubated ± anti-CD3 + anti-CD28 for 48 h, and percentage of cells staining positive for IL-17 was assessed by intracellular flow cytometry. **, P < 0.01. n.s. not statistically significant. (D and E) Histological evaluation of *C. albicans* infection in IL-17RA[KO] or IL-22[KO] mice. At day 5, sections of tongue from the indicated mice were stained with PAS or H&E and viewed at 10X-40X magnification. Data are representative of two mice per strain. Scale bars indicate 100 microns. (F) Quantitation of histological evaluation of PMNs. Six sections per mouse from 2 mice per group were evaluated for the number of PMNs per microscopic field, either in the vicinity of fungal lesions or parallel areas of sham-infected tongue. Evaluators were blinded to the identity of the samples. * P < 0.05. (G) IL-12[KO] but not IL-17RA[KO] mice can recover from oral *C. albicans* infection. WT (*n* = 4), IL-17RA[KO] (*n* = 12) and IL-12[KO] (*n* = 12) mice were infected with *C. albicans*. On the indicated days (ranging from 1–17), 1–3 mice per group were killed and *C. albicans* CFU per gram of tongue was evaluated by colony enumeration. Geometric mean for each day is shown. Time course data are from one experiment. (H) Weight loss correlates with degree of OPC susceptibility. Weight changes in IL-12[KO] and IL-17RA[KO] mice over time are shown.

This is unlikely because of subtle strain differences, as we observed no quantitative or qualitative differences in susceptibility to OPC between BALB/c and C57BL/6 (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20081463/DC1). Instead, IL-17 from another source such as NKT cells (32, 33) or neutrophils (29) may contribute to defense against OPC.

Downstream effects of IL–17RA signaling on saliva and oral mucosal tissue

To define mechanisms by which IL-17 mediates anti–*C. albicans* host defenses in the oral mucosa, we performed gene profiling analysis of tongue mRNA from WT or IL-17RA^{KO} mice at baseline (uninfected, "Day 0") or 24 h after infection ("Day 1"). Although WT mice showed no evidence of OPC 5 d after inoculation with *C. albicans* (Fig. 1), many immune genes were induced in WT oral mucosa after 24 h of exposure to *C. albicans*. Highly represented were "Th17 signature" genes, i.e., genes associated with Th17 function or known targets of Th17 hallmark cytokines (10, 34) (Fig. 5 A; see also Fig. S3). Among the most strongly induced genes were S100A8 and S100A9 (237- and 220-fold, respectively), CXCL2 (234-fold), CXCL5 (164-fold), LCN2/24p3 (lipocalin 2; 94-fold), IL-6 (41-fold), MMP8 (23-fold), CCL20 (29.5-fold), CSF3 (G-CSF, 17.8-fold), NF-κBiz (NF-κBζ; 12.3-fold), IL-22 (7-fold), and DEFB3 (BD3; 5.3-fold). All are known targets of IL-17 signal transduction, either alone or in combination with TNF-α or IL-22. Interestingly, several C-type lectin receptors were up-regulated that have been associated with *C. albicans* responses, including Clec4e (Mincle; 97.3-fold), Clec4n (Dectin-2; 11.9-fold), and Clec7a (Dectin-1; 9.2-fold) (35, 36) (Fig. 5 A). In contrast, few Th1- or Th2-related genes were up-regulated (see also Fig. S3). Quantitative real-time PCR experiments demonstrated that IL-17 was enhanced by approximately threefold in WT mice after *C. albicans* infection (Fig. 5 C). IL-22 was also up-regulated in the microarray and in real-time RT-PCR (Fig. 5 D). Messenger RNA encoding the Th1 cytokines IFN-γ and IL-12 were induced as well (Fig. 5, E and F), consistent with the fact that Th1-deficient (IL-12^{KO}) mice show an increased fungal burden, although no fungal lesions are manifest and almost no hyphal yeast cells are found invading the oral cavity in the IL-12^{KO} mice (Fig. 2 C).

In IL-17RA^{KO} mice, up-regulation of Th17 profile genes was considerably lower than in WT (Fig. 5 A, white bars vs. red bars; and Figs. S4–S6), although many were still induced to some extent, presumably via the activity of other remaining inflammatory cytokines. IL-17 was also constitutively elevated in IL-17RA^{KO} mice, which has been previously reported (8), as were IL-22, IFN-γ, and IL-12 (Fig. 5, D and F). When comparing differentially expressed genes in WT versus IL-17RA^{KO} mice 24 h after infection, there was a reduction in many genes associated with neutrophil recruitment such as CXCL1, CXCL5 (both 3.1-fold), and CSF3/G-CSF (3.3-fold; Fig. 5 B), consistent with the strik-

ing decrease in neutrophilic infiltrate observed in IL-23^{KO} and IL-17RA^{KO} mice compared with WT mice (Fig. 2).

In IL-17RA^{KO} mice compared with WT, there was a notable diminution in genes encoding antimicrobial proteins that are known to exert candidacidal activity, including BD3 (DEFB3; 6-fold) and CCL20 (2.4-fold; Fig. 5 B) (37, 38). Indeed, BD3 was the most differentially expressed gene between WT and IL-17RA^{KO} mice based on the microarray analysis (Fig. 5 B). Differential expression of BD3 and CCL20 mRNA between WT and IL-17RA^{KO} mice was verified by real-time RT-PCR (Fig. 5 H and Fig. 6 A). CCL20 and BD3 have both been reported to have direct killing activity against *C. albicans* (38). We verified that BD3 could indeed kill *C. albicans* cells in vitro comparable to physiological concentrations (7 μM) of a human salivary anticandidal peptide histatin-5 (37), but there was no detectable killing activity by recombinant CCL20, even at high concentrations (25 μM; Fig. 6 C).

Saliva contains potent antimicrobial factors, and patients with salivary gland defects caused by radiation or Sjogren's syndrome are highly susceptible to OPC (39, 40). Almost no biochemical characterization of murine saliva has been reported. As shown in Fig. 6 (A and B), WT mice express increased levels of BD3 mRNA and protein in tongue after *C. albicans* infection, which are markedly reduced in IL-17RA^{KO} mice. Next, we evaluated the ability of saliva from various KO mouse strains to kill *C. albicans* cells in vitro (Fig. 6, C and D). As a positive control for candidacidal activity, we verified that the human salivary peptide histatin-5 at a concentration of 7 μM killed ~22% of *C. albicans*, as previously reported (41) (Fig. 6, C and D). WT murine saliva was collected after carbachol injection and incubated with *C. albicans* cells in vitro, and yeast survival was determined by plating
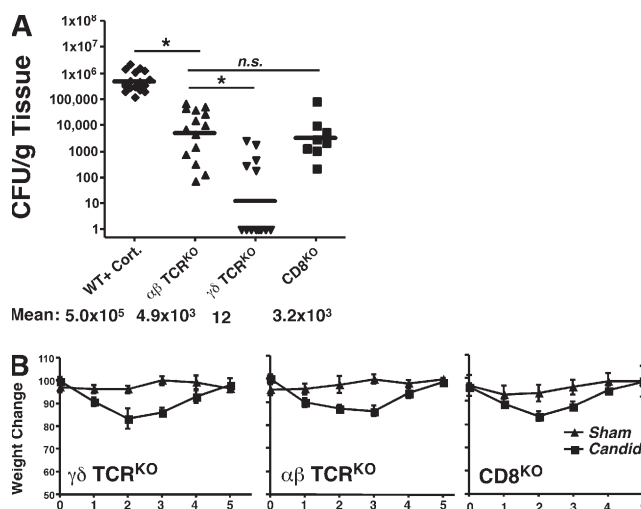
Mean: 5.0x10^5 4.9x10^3 12   3.2x10^3

Figure 4.   Host defense against oral *C. albicans* is mediated mainly by αβ-T cells, rather than γδ-T cells. Mice deficient in αβ-T cells (*n* = 14), γδ-T cells (*n* = 13), or CD8 (*n* = 8) were inoculated with *C. albicans* and fungal burden (CFU/g tissue) in tongue (A) and daily weight (B) were assessed. CD8^{KO} mice were evaluated in one experiment, and the γδ^{KO} and αβ^{KO} mice were evaluated in three independent experiments.

and colony enumeration. Saliva from WT mice killed a mean of 14.5% of *C. albicans* cells, and was not statistically different from saliva derived from IL–17RA–deficient mice (Fig. 6 D). However, saliva from IL–23[KO] mice reproducibly showed significantly reduced fungicidal activity (approximately three-fold). 24 h after oral inoculation with *C. albicans*, saliva from both IL–23[KO] and IL–17RA[KO] mice exhibited reduced candidacidal activity compared with WT saliva. In contrast, saliva from IL–12[KO] mice consistently showed elevated candidacidal effect compared with WT, both at baseline and 24 h after

inoculation (Fig. 6 D). Therefore, IL–23 and IL–17, but not IL–12, regulate the antimicrobial activity of mammalian saliva.

To determine whether IL–17 stimulates anticandidal activity on cells that form nonimmune tissue, we stimulated a murine stromal cell line with IL–17 and/or TNF-α and assessed conditioned media (CM) for effects on *C. albicans*. CM from cells treated with IL–17 or TNF-α alone did not trigger candidacidal activity, but CM from cells treated with both IL–17 and TNF-α killed ∼30% of yeast in this assay, indicating that these cytokines stimulate secretion of factors
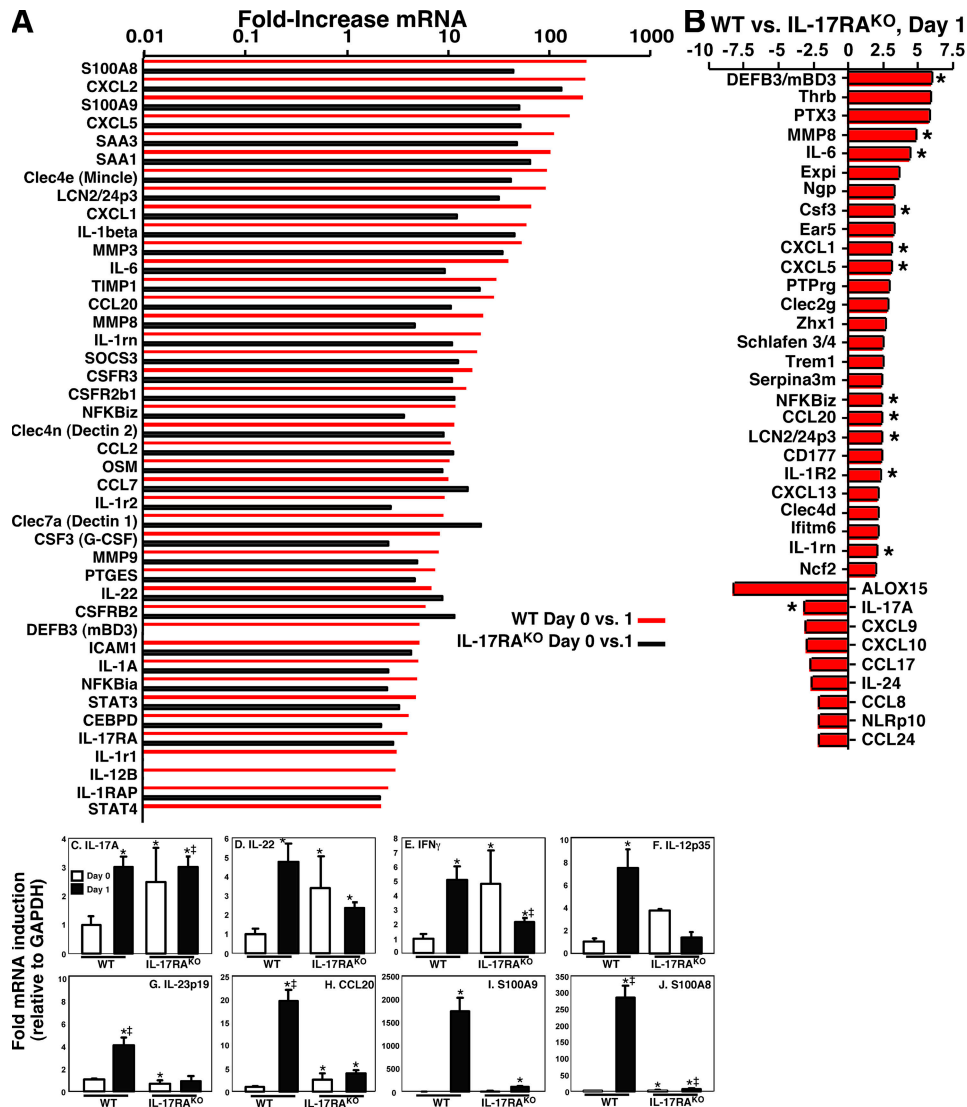

Figure 5. **Mechanism of IL–17–mediated host defense against OPC.** (A) Oral inoculation with *C. albicans* induces Th17 signature genes in oral mucosal tissue, which are suppressed in IL–17RA[KO] mice. Tongue tissue from female WT or IL–17RA[KO] mice (*n* = 2, from independent experiments) was taken at baseline (day 0) or 24 h after oral *C. albicans* inoculation (day 1), and cDNA was prepared and hybridized to Affymetrix genechips. Shown are Th17-related genes found to be induced on day 1 compared with day 0 from WT mice (red bars) or IL–17RA[KO] mice (white bars). (B) Differentially expressed genes in WT versus IL–17RA[KO] mice determined by microarray analysis. Fold change of genes induced in WT mouse tongue compared with IL–17RA[KO] tongue on day 1 are indicated. * indicates genes previously connected to IL–17 or Th17 cells. (C–J) OPC-induced expression of selected genes. Tongue mRNA from WT or IL–17RA[KO] mice on day 0 or day 1 (mRNA from two mice from independent experiments) was subjected to quantitative real-time RT-PCR analysis in triplicate. Data are presented as fold induction, normalized to GAPDH. *, P < 0.05 compared with WT Day 0; ‡, P < 0.05 compared with IL–17RA[KO] Day 0.

with antimicrobial activity (Fig. S2, available at http://www .jem.org/cgi/content/full/jem.20081463/DC1). Thus, in an inflammatory environment, IL-17 contributes to an antifungal state in cooperation with other inflammatory cytokines.

Th1 cells, but not Th17 cells, help limit breaching of the oral mucosal barrier

OPC generally remains contained in the oral cavity, and is not thought to breach this mucosal barrier to establish a dis-
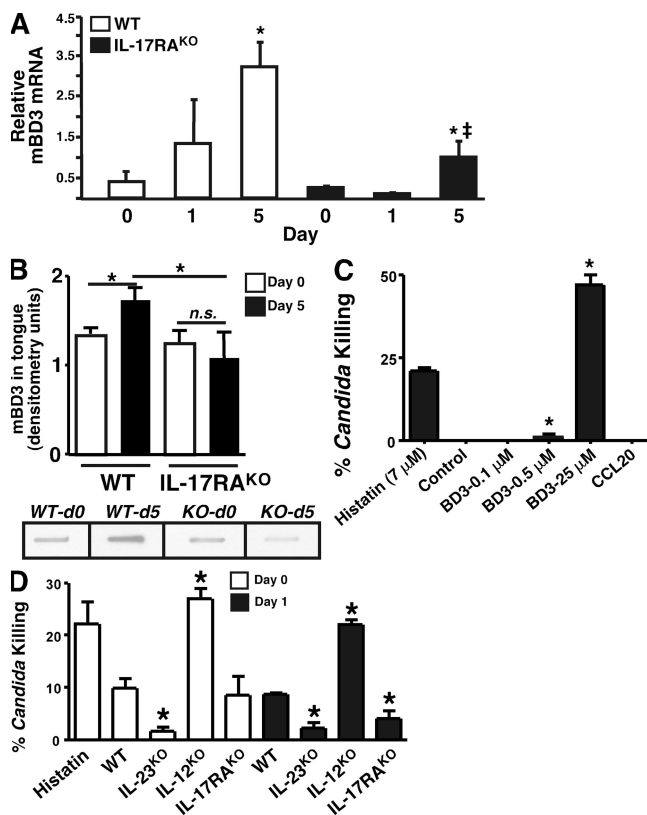
seminated form of disease (42). To determine whether Th1 or Th17 cells contribute to this immune barrier, we evaluated $C.$ $albicans$ CFU in kidney. Surprisingly, IL-12^{KO} mice, but not IL-23^{KO} or IL-17RA^{KO} mice, showed some evidence of kidney infection (Fig. 7). Consistently, mice lacking $\alpha\beta$-TCR cells (which lack Th1 cells and other Th subsets) showed evidence of systemic infection, although CFUs were not high enough to cause lethality. Nonetheless, these data suggest that Th1 cells, although relatively less important for host defense against OPC compared with Th17 cells, may be an important component of fungal immunity by helping to limit spread beyond the oral cavity. Still, because the fungal burden was sublethal and AIDS patients with low CD4$^+$ T cell counts do not generally contract disseminated candidiasis, additional components evidently contribute to controlling dissemination of $C.$ $albicans$.

DISCUSSION

OPC is a debilitating disease of immunodeficiency, characterized by fungal lesions on the palate, oropharynx, tongue, and buccal mucosa. Disease can progress to esophageal candidiasis, and limit nutritional intake and threaten overall health. OPC is the most frequent opportunistic fungal infection among HIV$^+$ individuals not receiving effective antiretroviral therapy (14), and it is also a problem in cancer patients on chemotherapy (43). Candidiasis occurs in 35% of Sjogren's syndrome patients (40), and the elderly, infants, and denture wearers are also at risk (2).

An intriguing feature of host defense against $C.$ $albicans$ is the anatomical compartmentalization associated with disease. For example, HIV$^+$ individuals experience OPC but do not show enhanced incidence of vaginal or disseminated candidiasis (3). Immunity to OPC involves antifungal salivary proteins, PMNs, CD8$^+$ cells and nonimmune cells such as oral keratinocytes, stromal cells, and epithelial cells (2, 14). Interactions
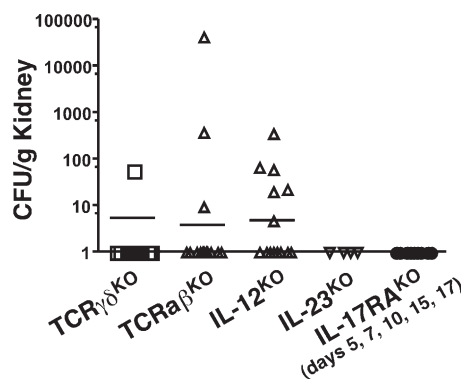

Figure 6. Anticandidal factors and Th17 deficiency. (A) IL-17RA^{KO} mice show decreased expression of mBD3 mRNA. Quantitative real-time RT-PCR analysis was performed in triplicate on tongue cDNA from WT or IL-17RA^{KO} mice ($n = 2$–4) at baseline (day 0) or day 1 or 5 after $C.$ $albicans$ infection. *, $P < 0.05$ compared with Day 0; ‡, $P < 0.05$ compared with WT sample at Day 5. (B) IL-17RA^{KO} mice show decreased expression of mBD3 protein. Tongue tissue from WT mice or IL-17RA^{KO} mice at day 0 or 5 ($n = 4$ for each condition) were applied to nitrocellulose in slot blots and probed with antibodies to mBD3. Signals were quantified by densitometry. *, $P < 0.05$. ns = not significant. Sample slot blot samples are shown below (note: all were taken from the same film at the same exposure level). (C) Candidacidal activity of BD3 and CCL20. Recombinant BD3 (0.1, 0.5 or 25 µM) and CCL20 (25 µM) were evaluated for fungicidal activity by incubation with 10^6 $C.$ $albicans$ cells for 1.5 h in triplicate. Human histatin-5 (7 µM) was used as a positive control (37). Yeast were diluted and spread on YPD agar for colony enumeration. Data were compared by unpaired Student's t test (*, $P < 0.05$ compared with WT controls). Data are representative of three independent experiments. (D) Reduced candidacidal activity in IL-17RA-deficient and Th17-deficient (IL-23^{KO}) saliva. Saliva was collected from the indicated mice ($n = 3$–6) after carbachol injection and assayed for candidacidal activity in triplicate as in B. Experiments were performed twice with similar results.

Figure 7. IL-12, but not IL-23 or IL-17RA, limit dissemination of $C.$ $albicans$ from the oral cavity. The indicated KO mice ($\gamma\delta$-T^{KO}, $n = 13$; $\alpha\beta$-T^{KO}, $n = 14$; IL-12^{KO}, $n = 14$; IL-23^{KO}, $n = 4$; IL-17RA^{KO}, $n = 9$) were infected orally with $C.$ $albicans$ as in Fig. 1, and kidneys were isolated on day of sacrifice. After homogenization, CFU/gram kidney was determined by colony enumeration in triplicate. Data were collected from all experiments in Figs. 2–4.

between PMNs and T cells are required for maintaining host defense to OPC (2, 44). Th17 cells link CD4 cells and the PMN response by IL–17–mediated regulation of neutrophil expansion, recruitment, and migration (15, 29), and provide a satisfying explanation for why CD4$^+$ T cells are so important in OPC. Consistently, we observed a large neutrophilic infiltrate in resistant mice (WT, IL–12^{KO}, and IL–22^{KO}) after *C. albicans* inoculation, which was greatly diminished in susceptible (IL–23^{KO} and IL–17RA^{KO}) mice (Figs. 2 and 3).

Gene-targeted mice permit hypothesis-driven studies to define immune components involved in candidiasis. Huang et al. (17) identified a protective role for IL–17 in disseminated candidiasis, as IL–17RA^{KO} mice are susceptible to systemic disease caused by defects in neutrophil migration and chemokine expression. Dectin-1^{KO} mice show defective development of Th17 cells (18, 19) and are susceptible to disseminated candidiasis, although this finding has been controversial (19, 45). Conversely, IL–23 and IL–17 drive inflammatory pathology in models of pulmonary aspergillosis and gastric candidiasis (22), and IL–17 neutralization unexpectedly protects from disseminated candidiasis in a study by this group (46). Although the relevance of the gastric candidiasis model to human disease is questionable, Th17 cells mediate pathology in airway disease, rheumatoid arthritis, and EAE (47, 48). Recent studies support the notion that the Th17 pathway, rather than the Th1 pathway, drives defense to OPC. IFN-γ^{KO} and IL–4^{KO} mice are resistant to OPC, whereas IL–12p40^{KO} mice are susceptible (24). Here, we demonstrate that IL–23 and IL–17RA are required for effective resistance to OPC, whereas IL–12 and IL–22 play less critical roles (Figs. 2 and 3). The propensity of proinflammatory cytokines to play opposing roles in disease is well documented (49), and IL–17 appears to be no exception. For example, in the oral cavity, the role of IL–17 in periodontal disease may be host-protective or destructive depending on disease severity and chronicity (15, 50).

Our data indicate that γδ-T cells, although a major source of IL–17 (29), are less important than αβ-T cells in preventing OPC (Fig. 4). This was surprising, given the rapid time course of disease (5 days). However, only 2% of T cells in the oral epithelium are γδ-T cells (51), and these cells apparently do not play a defensive role in vaginal candidiasis (52). γδ-T cells also produce IL–22, which we find to play a relatively less important role in OPC (Fig. 3 A). On the other hand, γδ-T cells have been suggested to enhance resistance to gastric candidiasis (53). We have noticed that some γδ-T cell–deficient mice experienced higher levels of *C. albicans* CFU when experiencing stress (e.g., poor recovery from anesthesia or the presence of a dead mouse in the cage; unpublished data). Although mice were eliminated from the study when such confounding factors were obvious, this may explain why a subset of γδ-T cell–deficient mice showed detectable *C. albicans* colonization (Fig. 4). Unidentified environmental stressors may have predisposed certain γδ-TCR^{KO} mice to OPC, and thus the γδ T cell subset may provide an additional layer of protection against candidiasis.

Saliva plays a vital defensive role in the oral cavity (14). Saliva from IL–17RA^{KO} and IL–23^{KO} is defective in killing *C. albicans* (Fig. 6 D), which may be at least partly caused by a deficiency in antimicrobial substances (Fig. 5). BDs are produced by salivary glands but also by epithelial cells in the gingiva, buccal mucosa, and tongue (54), and they exert anticandidal activity in vitro (Fig. 6; Fig. 5 B; and Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20081463/DC1) (37, 55). IL–17 regulates BD3 in lung cells in humans and mice (56). Here, we show that BD3 mRNA and protein are up-regulated in WT mice after infection with *C. albicans*, and there was an impairment in BD3 expression in IL–17RA^{KO} tongue compared with WT mice (Fig. 5 B; Fig. 6, A and B; and Figs. S3–S6). In contrast to tongue, there was little difference in BD3 levels in saliva between WT and IL–17RA^{KO} mice (unpublished data). Thus, although the basis for the reduced salivary fungicidal activity in IL–23^{KO} and IL–17RA^{KO} remains unclear, BD3 likely contributes to the reduced infection on the oral mucosal surface.

Antimicrobial peptides also function as chemotactic factors for immune cells. BD2 recruits dendritic cells and T cells via CCR6 (57), and S100A8/A9 are chemotactic for neutrophils (58). Another ligand for CCR6 is CCL20, which was impaired in IL–17RA^{KO} mice after infection (Fig. 5 H). Like BDs, CCL20 has chemotactic activity for T cells and DCs. CCL20 is regulated by IL–17 (59, 60), is expressed by Th17 cells (61) and has been linked to Th17 recruitment to inflamed sites (13). Although CCL20 has been reported to exert antimicrobial activity against *C. albicans* in vitro (38), we did not observe this effect, perhaps because of differences in the commercial preparations of the chemokine or the specific strain of *C. albicans* used (Fig. 6 C).

Our findings are similar to, yet distinct from, observations made regarding Th17 function at other mucosal sites. A microarray study of *K. pneumonia*–infected lung found many of the same genes, including BDs, CXC chemokines, lipocalin 2, and IL–6, although BD3 was not identified in that study (27). Microarray studies in RA synoviocytes (60) and after *Salmonella enterica* serovar Typhimurium infection in macaques (9) found similar IL–17 target genes induced during disease states. The *K. pneumonia* study reported that IL–22, another Th17 cytokine, is a critical host-protective cytokine (27), but our data suggest that IL–22 is less important than IL–17 in the oral cavity (Fig. 3 A). Humans with mutations in STAT3 (hyper-IgE syndrome [HIES]) fail to develop Th17 cells and experience recurrent oral and mucocutaneous *C. albicans* infections. One study indicated that HIES patients show normal levels of IL–22 in the CD4$^+$ T cell population (20). Conversely, several other studies report reduced IL–22 levels in HIES patients (62–64). Because IL–17 and IL–22 cooperatively regulate chemokines and antimicrobial genes (65), we cannot rule out a role for IL–22 in contributing to host defense to OPC, particularly in humans.

Intriguingly, IL–12, and hence Th1 cells, may help limit transition to a disseminated form of candidiasis, as IL–12^{KO} but not IL–23^{KO} or IL–17RA^{KO} exhibited detectable *C. albicans*

infection in kidney (Fig. 7). Although OPC causes considerable morbidity, disseminated candidiasis is associated with ~40% mortality and is the fourth most common pathogen isolated from hospitalized patients (66). It is generally thought that disseminated candidiasis is initiated when *C. albicans* enters the bloodstream from the gut or indwelling catheters (42, 67). This study is, to our knowledge, the first to demonstrate that oral disease can lead directly to systemic candidiasis, and moreover that Th1 cells are important in preventing a breach in the mucosal barrier. However, the kidney fungal burden in IL-12[KO] mice was low, and thus other mechanisms contribute to effective barrier function. This contrasts a recent study in the gut, which found that selective depletion of Th17 cells is seen after SIV infection in macaques, and thus promotes dissemination of *Salmonella* (9, 68). Finally, IL-12p35 was recently shown to be part of the cytokine IL-35 produced by regulatory T cells (69), which could conceivably play a role in barrier function by limiting inflammatory tissue damage during oral infection. Similarly, IL-17RA was recently reported to be a receptor subunit for the IL-25, which promotes Th2 cells (70).

Defining cytokines involved in immunity to OPC is important clinically, given increasing use of anticytokine biologics. The role of the IL-23 and IL-17 in autoimmunity has stimulated development of blocking agents for these cytokines (71). Although likely to prove useful in ameliorating inflammatory pathology, consequences to opportunistic infection need to be considered. Anti–TNF-α therapy predisposes to *M. tuberculosis* infection, histoplasmosis, and fungal pneumonia, but thrush is only rarely seen (72). Consistently, TNF-α[KO] mice only show a mild increase in susceptibility to OPC (24), but are sensitive to disseminated candidiasis (73). Our data and findings in HIES patients (20) suggest that complete IL-17 and IL-23 blockade could increase OPC susceptibility, but this will need to be evaluated in clinical settings.

In summary, immunity to *C. albicans* depends on multiple immune components, as well as anatomical location. This study shows unequivocally that IL-17 and Th17 cells play a key role in protecting the host against OPC, whereas Th1 cells and IL-22 play a relatively less important role.

MATERIALS AND METHODS

Mouse model of OPC. IL-17RA[KO] mice were a gift from Amgen, and IL-23p19[KO] mice were provided by Genentech. IL-22 KO mice were produced in a collaboration between Genentech and Lexicon Pharmaceuticals to analyze the function of 500 secreted and transmembrane proteins (74). All other mice were purchased from The Jackson Laboratory. All mice were on the C57BL/6 background, and all were age and sex matched. Mice were caged individually after infection. If indicated, mice were treated with 225 mg/kg cortisone acetate (Sigma-Aldrich) and infected under anesthesia by placing a 0.0025-g cotton ball saturated with 2×10^7 CFU *C. albicans* (CAF2-1) sublingually for 75 min, as previously described (25). No antibiotics were administered. Saliva was collected into chilled tubes after carbachol injection (100 μl at 10 mg/ml) and used immediately in candidacidal assays. All protocols were approved by SUNY Buffalo Institutional Animal Care and Use Committee.

***C. albicans* culture and handling, candidacidal assays.** *C. albicans* was cultured in YPD by standard methods. Candidacidal assays were generally performed as previously described (41). For saliva testing, 90 μl of freshly collected murine saliva was incubated with 10^4 cells of *C. albicans* for 1 h at 37°C, plated in triplicate, and assayed for colony enumeration. BD3 and cytokines were from R&D Systems.

Cell culture, flow cytometry, and histology. ST2 cells were stimulated with 100 ng/ml IL-17 or 2 ng/ml TNF-α for 24 h in α-MEM media supplemented with 10% FCS. Cytokines and defensins were obtained from R&D Systems. For FACS, cLNs were harvested at day of sacrifice and cells stimulated with α-CD3 and α-CD28 antibodies (BD) for 48 h. Samples were stained intracellularly with anti–IL-17-PE antibodies (BD) and analyzed on a FACSCalibur with CellQuest software. Paraffin-embedded samples were stained with hematoxylin and eosin or periodic-acid Shiff (PAS) by the University at Buffalo Histology Core. Sections were analyzed at 10–40X magnification, and all scale bars indicate 100 μm.

Microarray and real-time RT-PCR. Whole tongues from WT or IL-17RA[KO] female mice, age 10–16 wk ($n = 2$, age-matched within infection pairs), were harvested at baseline or 24 h after *C. albicans* inoculation and preserved in RNALater (Ambion). Total mRNA and cDNA were prepared and hybridized on Affymetrix Mouse 430–2 Genechips. Preparation and analysis were performed by the Roswell Park Cancer Institute Gene Expression Resource Facility (Buffalo, NY) using the Partek Genomics Suite 6.4 (RMA algorithm) with a quantile normalization and probe set summarization at median Pol. Real-time RT-PCR was performed on tongue samples in triplicate using SYBR Green and normalizing to GAPDH as previously described (75). Primers were purchased from SuperArray.

BD slot blot. Tongues from indicated mice were homogenized in lysis buffer (10 mM NaPB, 1 mM EDTA, 1 μg/ml aprotinin, leupeptin, pepstatin-A, 1 mM PMSF, and 0.1% SDS), centrifuged to remove debris, and quantified for protein content. A 1.2- μg sample was loaded per slot blot well onto nitrocellulose, and blots were probed with 1 μg/ml anti-BD3 antibodies (Santa Cruz Biotechnology). Blots were quantified by scanning densitometry on an HP Scanjet 3970 and analyzed with Quantity One software (BioRad Laboratories).

Statistics. At least two biological replicates were performed for all experiments unless otherwise indicated. Data were compared by Mann-Whitney or unpaired Student's *t* test using GraphPad Prism (v. 4) or Microsoft Excel software. P values < 0.05 were considered significant.

Online supplemental material. Fig. S1 shows that similar levels of *C. albicans* infection are found in IL-17RA–deficient mice on the C57BL/6 background compared with the BALB/c background. Fig. S2 shows that IL-17 and TNF-α stimulation of a murine bone marrow stromal cell line (ST2) triggers secretion of factors with significant candidacidal activity. Figs. S3–S6 show primary microarray data comparing gene expression in tongue tissue of female WT and IL-17RA[KO] mice either at baseline or 24 h after *C. albicans* inoculation. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20081463/DC1.

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