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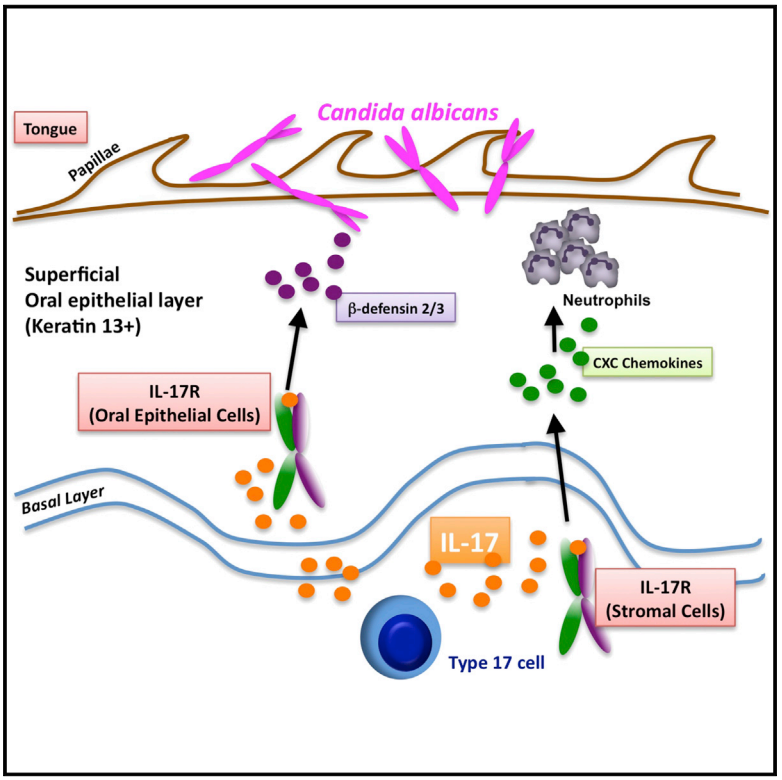
IL-17 Receptor Signaling in Oral Epithelial Cells Is Critical for Protection against Oropharyngeal Candidiasis

Graphical Abstract



Highlights

- Human and murine oral epithelial cells show concordant responses to *C. albicans*
- IL-17R signaling in the oral epithelium is necessary for defense against candidiasis
- IL-17R signaling in oral epithelial cells is a major driver of β-defensin expression
- β-defensin-3-deficient mice are susceptible to oral candidiasis

Authors

Heather R. Conti, Vincent M. Bruno, Erin E. Childs, ..., Jay K. Kolls, Satrajit Sinha, Sarah L. Gaffen

Correspondence

heather.conti@utoledo.edu (H.R.C.), sarah.gaffen@pitt.edu (S.L.G.)

In Brief

IL-17 receptor signaling is required to prevent oropharyngeal candidiasis ("oral thrush") in both mice and humans. Conti et al. demonstrate in mice that IL-17R-dependent antifungal responses in superficial oral epithelial cells (OECs) are critical for protection. Moreover, OECs dominantly control IL-17R-dependent responses through production of the antimicrobial peptide β-defensin 3.

Conti et al., 2016, Cell Host & Microbe *20*, 606–617
November 9, 2016
http://dx.doi.org/10.1016/j.chom.2016.10.001

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IL-17 Receptor Signaling in Oral Epithelial Cells Is Critical for Protection against Oropharyngeal Candidiasis

Heather R. Conti,[1,2,*] Vincent M. Bruno,[3] Erin E. Childs,[1] Sean Daugherty,[3] Joseph P. Hunter,[2] Bemnet G. Mengesha,[2] Danielle L. Saevig,[2] Matthew R. Hendricks,[1] Bianca M. Coleman,[1] Lucas Brane,[1] Norma Solis,[4] J. Agustin Cruz,[1] Akash H. Verma,[1] Abhishek V. Garg,[1] Amy G. Hise,[5,6] Jonathan P. Richardson,[7] Julian R. Naglik,[7] Scott G. Filler,[4] Jay K. Kolls,[8] Satrajit Sinha,[9] and Sarah L. Gaffen[1,7,10,*]

[1]Division of Rheumatology and Clinical Immunology, University of Pittsburgh, Pittsburgh, PA 15261, USA
[2]Department of Biological Sciences, University of Toledo, Toledo, OH 43606, USA
[3]Institute for Genome Sciences, University of Maryland School of Medicine, Baltimore, MD 21201, USA
[4]Division of Infectious Diseases, Los Angeles Biomedical Research Institute, Harbor-UCLA Medical Center, Torrance, CA 90502, USA
[5]Department of Pathology, Case Western Reserve University, Cleveland, OH 44106, USA
[6]Louis Stokes Cleveland Veterans Affairs Medical Center, Cleveland, OH 44106, USA
[7]Department of Mucosal and Salivary Biology, King's College London, London SE1 1UL, UK
[8]Richard King Mellon Foundation for Pediatric Research, Children's Hospital of UPMC, Pittsburgh, PA 15224, USA
[9]Department of Biochemistry, University at Buffalo, State University of New York, Buffalo, NY 14203, USA
[10]Lead Contact
*Correspondence: heather.conti@utoledo.edu (H.R.C.), sarah.gaffen@pitt.edu (S.L.G.)
http://dx.doi.org/10.1016/j.chom.2016.10.001

SUMMARY

Signaling through the IL-17 receptor (IL-17R) is required to prevent oropharyngeal candidiasis (OPC) in mice and humans. However, the IL-17-responsive cell type(s) that mediate protection are unknown. Using radiation chimeras, we were able to rule out a requirement for IL-17RA in the hematopoietic compartment. We saw remarkable concordance of IL-17-controlled gene expression in *C. albicans*-infected human oral epithelial cells (OECs) and in tongue tissue from mice with OPC. To interrogate the role of the IL-17R in OECs, we generated mice with conditional deletion of IL-17RA in superficial oral and esophageal epithelial cells ($Il17ra^{\Delta K13}$). Following oral *Candida* infection, $Il17ra^{\Delta K13}$ mice exhibited fungal loads and weight loss indistinguishable from $Il17ra^{-/-}$ mice. Susceptibility in $Il17ra^{\Delta K13}$ mice correlated with expression of the antimicrobial peptide β-defensin 3 (BD3, *Defb3*). Consistently, $Defb3^{-/-}$ mice were susceptible to OPC. Thus, OECs dominantly control IL-17R-dependent responses to OPC through regulation of BD3 expression.

INTRODUCTION

Fungal infections are an increasing threat (Brown et al., 2012). To date, there are no licensed vaccines to any fungal pathogens, and antifungal medications are costly, toxic, and increasingly ineffective due to drug resistance. Oropharyngeal candidiasis (OPC; thrush) is an opportunistic infection caused by the commensal fungus *Candida albicans*. Susceptibility is associated with T cell deficiency, as most HIV/AIDS patients experience recurrent OPC. Thrush is also associated with denture use, salivary gland defects (e.g., Sjögren's syndrome), and immunosuppressive regimens associated with cancer, transplantation, or autoimmunity. Oral thrush also causes nutritional deficits in infants and a failure to thrive (Fidel, 2011; Glocker and Grimbacher, 2010).

IL-17 (IL-17A) is a vital mediator of antifungal immunity. IL-17 is expressed by lymphocytes, most notably $CD4^+$ "Th17" cells, but also $CD8^+$ T cells and various innate immune populations such as γδ-T cells, natural killer T (NKT) cells, "natural" Th17 cells, and innate lymphoid 3 cells (ILC3s) (Cua and Tato, 2010). Some data suggest that IL-17 may be expressed by myeloid cells, though this remains controversial (Huppler et al., 2015; Taylor et al., 2014). Humans with defects in the IL-17R signaling pathway show remarkably restricted disease susceptibility, with high sensitivity to chronic mucosal candidiasis (CMC) and *S. aureus* infections, but typically not to other infections (Milner and Holland, 2013). Like immunocompetent humans, wild-type (WT) mice are resistant to OPC (Kamai et al., 2001). However, $Il17ra^{-/-}$, $Il17rc^{-/-}$, $Il23^{-/-}$ $Act1^{-/-}$, and $Rorc^{-/-}$ mice are all susceptible (Conti et al., 2009, 2014; Ferreira et al., 2014; Ho et al., 2010), pointing to a role for the IL-17/Th17 pathway in OPC.

The oral mucosa is poorly understood with respect to immune function. A key constituent of oral immunity is the oral epithelial cell (OEC), which mediates early recognition of and response to microbes (Moyes et al., 2015). Like other mucosae, the oral epithelium constitutes a physical barrier to restrict pathogen entry. OECs interact with *C. albicans* through multiple receptors, including HER2/Neu and EGFR (Zhu et al., 2012). OECs also sense the dimorphic transition from the commensal yeast to virulent hyphae by inducing distinct downstream signaling pathways (Moyes et al., 2016). In response to *C. albicans*, OECs express innate cytokines and antimicrobial effectors, including IL-1β,

CCL20, G-CSF, and β-defensins (BDs). Within 1 day of infection, IL-17 is highly expressed in lymphocytes (Conti et al., 2014; Hernández-Santos et al., 2013). In mice, which do not harbor *C. albicans* as a commensal microbe and thus are immunologically naive to this organism, IL-17 is produced upon first encounter by innate lymphocytes, specifically γδ-T cells and $CD4^+TCR\beta^+$ "natural" Th17 populations (Conti et al., 2014; Huppler et al., 2015). In recall settings, IL-17 is additionally made by conventional Th17 cells, which augment the innate type 17 response to improve *C. albicans* clearance (Bär et al., 2012; Hernández-Santos et al., 2013).

IL-17 signaling induces a panel of antifungal target genes that collectively control C. *albicans* from the oral cavity. Downstream gene products include neutrophil-recruiting factors (CXC chemokines and G-CSF), myeloid and lymphoid chemoattractants (CCL20 and MCP1), and antimicrobial peptides (AMPs; BDs, lipocalin-2, and S100A8/9) (Conti and Gaffen, 2015). While IL-17-dependent signals are essential for effective immunity to OPC, the nature of the responding cell type(s) is less clear. OECs do not express IL-17 (Huppler et al., 2015) but do express the IL-17R (Gaffen et al., 2014). The oral mucosa is enriched for hematopoietic cells during infection, which also express high levels of IL-17RA (Ishigame et al., 2009; Ye et al., 2001). In this regard, a recent study reported that IL-17RA signaling on NK cells mediates immunity to bloodstream *C. albicans* infections (Bär et al., 2014). Additionally, saliva is an important component of immune control of *C. albicans*, and salivary gland epithelial cells also respond to IL-17 to produce salivary AMPs (Conti et al., 2011).

The objective of this study was to identify the key IL-17-responding cell(s) in OPC. We approached this question with radiation chimeras and by creating CRE-expressing transgenic mice with specificity for suprabasal oral/esophageal epithelium. Whereas IL-17RA-mediated responses in the hematopoietic compartment were dispensable for immunity to OPC, a deficiency of IL-17RA in OECs rendered mice almost as susceptible as a complete *Il17ra* knockout. Gene profiling showed close parallels between human OECs infected in vitro with *C. albicans* and genes induced in murine oral tissue during OPC. In contrast to $Il17ra^{-/-}$ mice, regulation of neutrophil-specific genes was largely intact in mice with the conditionally targeted *Il17ra* allele, whereas expression of BD3 was markedly impaired. Mice lacking BD3 were just as susceptible to OPC as $Il17ra^{-/-}$ mice. Thus, the OEC is the dominant IL-17-responsive cell type in controlling immunity to oral mucosal candidiasis.

RESULTS

IL-17RA on Hematopoietic Cells Is Dispensable for Protection from OPC

We evaluated the contribution of IL-17RA in hematopoietic cells in OPC using an adoptive transfer approach. Femoral bone marrow (BM) cells (10^3) from $Il17ra^{-/-}$ ($CD45.2^+$) and WT ($CD45.1^+$) mice were transferred into lethally irradiated $Il17ra^{-/-}$ or WT recipients. After 8 weeks, reconstitution was confirmed by flow cytometry of peripheral blood (data not shown). Successfully reconstituted mice were subjected to OPC by sublingual incubation with a cotton ball soaked in 10^7 colony-forming units (CFU)/mL *C. albicans* yeast (Solis and Filler, 2012). After 5 days, oral fungal loads were assessed by CFU enumeration, which has a limit of detection (LOD) of ~30 CFU/g (Whibley et al., 2016). WT mice reconstituted with WT or $Il17ra^{-/-}$ BM were resistant to infection, with a negligible fungal load (CFU < 2), indicating that IL-17RA expression on hematopoietic cells is not required for fungal clearance (Figure 1A). As previously shown, $Il17ra^{-/-}$ mice were susceptible to OPC (Conti et al., 2009), displaying a mean fungal burden of 1,847 CFU/g. Similarly, $Il17ra^{-/-}$ mice reconstituted with WT or $Il17ra^{-/-}$ BM exhibited fungal loads that were indistinguishable from $Il17ra^{-/-}$ mice (3,635 and 2,153 CFU/g, respectively). Consistently, WT recipients returned to starting weight by day 5, regardless of BM source. In contrast, $Il17ra^{-/-}$ mice experienced progressive weight loss, even when reconstituted with WT BM (Figure 1B).

Exposure to *C. albicans* activates a reproducible gene profile in mouse oral tissue, which peaks at day 2, when fungal loads are highest. Many of these genes are impaired in $Il17ra^{-/-}$ mice, such as *Defb3* (encoding BD3) (Conti et al., 2009; Hernández-Santos et al., 2013). In keeping with their fungal loads, WT mice receiving WT or $Il17ra^{-/-}$ BM induced *Defb3* efficiently. However, induction was lower in $Il17ra^{-/-}$ recipients reconstituted with WT BM. Thus, IL-17RA is required exclusively in non-hematopoietic cells.

C. albicans Induces an Antifungal Gene Response in Human OECs

Signals induced in OECs upon early exposure to *C. albicans* help distinguish between commensal colonization and pathogenic invasive infections (Moyes et al., 2015). In an effort to understand how IL-17 signaling impacts the response of human OECs to *C. albicans* infection, OKF6/TERT-2 oral keratinocytes were treated with IL-17A (50 ng/mL) and a suboptimal dose of TNFα (0.5 ng/mL) for 24 hr. Cells were then infected with 1×10^7 *C. albicans* yeast cells for 5 hr and subjected to RNA sequencing (RNA-seq). A total of 358 genes exhibited a minimum 2-fold change in expression ($p < 0.05$) between the TNF/IL-17A-treated and untreated OKF6/TERT-2 monolayers (Figure 2A; Table S1, available online). Many of the genes exhibiting TNF/IL-17-sensitive expression in OECs have mouse homologs that are IL-17 dependent in OPC (Conti et al., 2009). These included chemokines, cytokines, transcriptional or post-transcriptional immune regulators, AMPs, and other factors associated with inflammation.

Insights into pathogenesis can be obtained by inferring signal transduction pathways based on downstream gene expression changes, a particularly useful way to compare species where not all genes have direct homologs (Liu et al., 2015). Accordingly, we compared the human OEC RNA-seq dataset to an RNA-seq profile of WT and $Il17ra^{-/-}$ mice 24 hr after oral infection (Tables S1 and S2). Data were analyzed using the Upstream Regulator Analytic from Ingenuity Pathway Analysis software, which assesses the overlap between empirical data and a curated database of target genes for each of several hundred known regulatory proteins. A number of signaling pathways were identified common to both datasets, including TNF- and IL-17-based pathways, TLR pathways, NF-κB activation, and EGFR signaling, among others (Figure 2B). Thus, despite the substantial differences between the experimental systems (human versus mouse, culture monolayers versus tissue, cytokine treatment versus genetic deletion), data confirm that IL-17 signaling

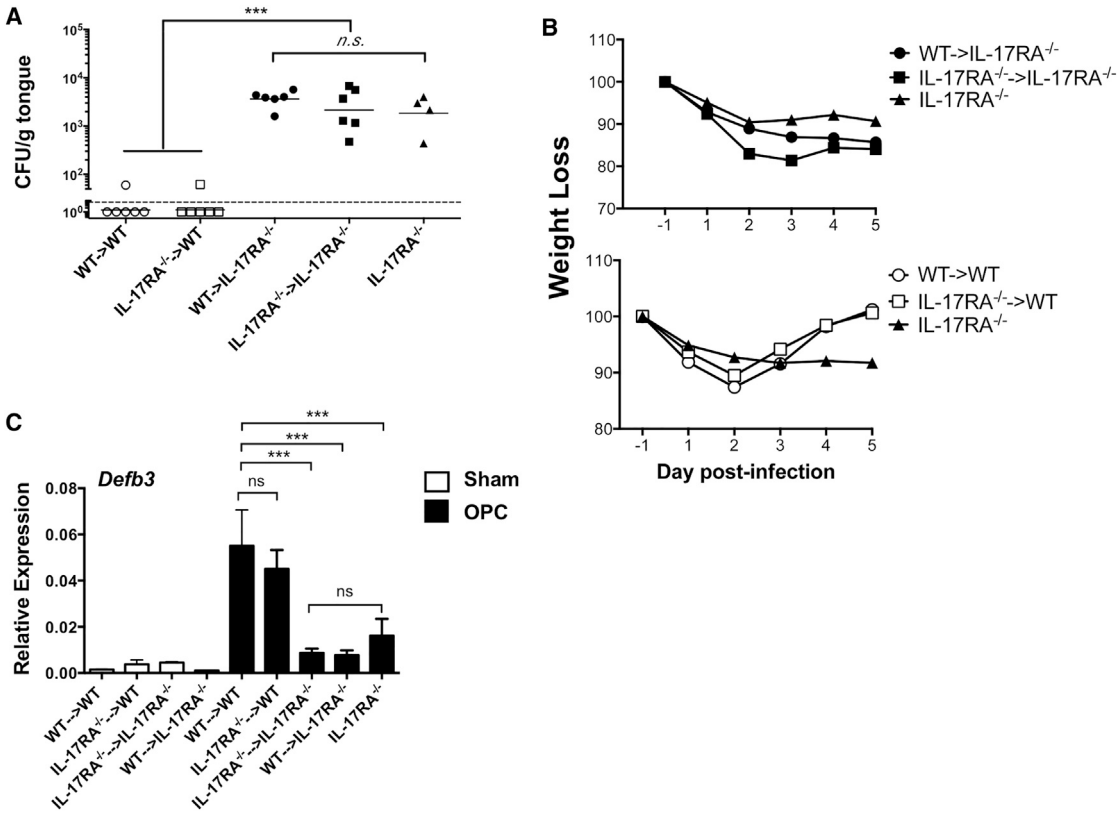

Figure 1. IL-17RA Expressed on Hematopoietic Cells Is Dispensable for Protection against Oral Candidiasis

Reciprocal adoptive transfers of femoral BM were performed in WT or *Il17ra*$^{-/-}$ mice followed by oral infection with *C. albicans*.

(A) After 5 days, fungal loads were assessed by CFU enumeration of tongue homogenates. Bars show geometric mean. Each point is one mouse. ***$p < 0.01$ by ANOVA and t test with Mann-Whitney correction.

(B) Weight was assessed daily and is shown as mean percent of starting weight.

(C) *Defb3* was evaluated on day 5 by qPCR. Data relative to *Gapdh* + SEM. ***$p < 0.01$ by ANOVA and post hoc Tukey's test. N.S., not significant. All experiments were performed at least twice.

in human OECs parallels IL-17-dependent gene expression during OPC.

Generation of Cre-Expressing Transgenic Mice with Specificity for Oral Epithelium

To date, there are surprisingly few genetic tools available to interrogate activity in oral epithelium. The oral mucosa consists of morphologically discrete epithelial layers with keratinization patterns distinct from skin (Presland and Dale, 2000). Whereas keratin 14 (K14) and K5 are expressed in the basal stem cells of oral keratinized and non-keratinized epithelia, K13 is expressed superficially in the differentiated suprabasal layers (BLs), representing the first line of contact to oral microbes. To identify K13 regulatory sequences, we cloned the *Krt13* putative proximal promoter (−3,070 to +50 relative to the transcriptional start site) upstream of luciferase. This construct exhibited potent activity in the murine oral keratinocyte (IMOK) cell line (Figure 3A) (Parikh et al., 2008). To evaluate the *Krt13* promoter in vivo, we created transgenic mice expressing *LacZ* driven by the K13 promoter (K13^{LacZ}). Oral tissues were analyzed for β-galactosidase activity by Xgal staining, including esophagus, buccal mucosa, and dorsal and ventral tongue. K13-driven *LacZ* activity was restricted to the differentiated BLs of these tissues, with no discernible expression in the basal or sub-basal layers (Figure 3B). There was no *LacZ* activity in gut, fore-stomach, or gastrointestinal (GI) tract (data not shown). Therefore, the K13 promoter shows specificity for expression in OECs, consistent with its known expression pattern.

We created transgenic mice expressing the K13 promoter upstream of a *Cre* recombinase cassette (Figure 3C). Four PCR^{+} founders were identified, and tissues were evaluated for coincident *Cre* and *Krt13* mRNA expression in tongue and esophageal tissue (Figure 3D). The line where *Cre* expression most closely paralleled the known expression pattern of *Krt13* (and K13-LacZ mice) was selected for all subsequent experiments. Progeny from *Cre*-expressing lines were then crossed to *Gt(Rosa)26*^{tm1Sor} reporter mice (K13^{CRE}*Rosa*26^{LacZ}). LacZ staining of sections from the K13^{CRE}-*Rosa*26^{LacZ} mice showed strong Cre expression and activity in oral tissues such as the tongue and buccal mucosa, as well as the upper GI tract, including stomach (Figure 3E) and the vaginal epithelium (data not shown). Little or no LacZ was detected in skin, submandibular salivary gland, or hematopoietic organs such as spleen and cervical lymph nodes (cLN).

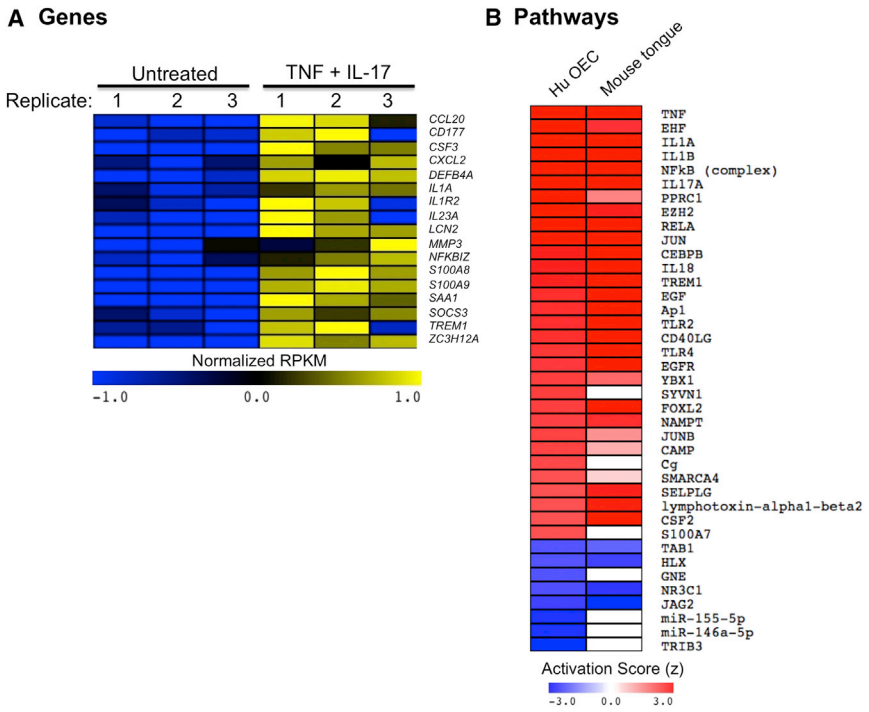

Figure 2. *C. albicans* Induces an Antifungal Gene Response in Human OECs that Parallels the IL-17-Dependent Response to Murine OPC

(A) OKF6/TERT2 human OECs were treated with IL-17+TNFα for 24 hr. Cells were exposed to *C. albicans* yeast for 5 hr and subjected to RNA-seq. Data show normalized RPKM values of genes whose orthologs were previously shown to be induced in an IL-17R-dependent manner in murine OPC (Conti et al., 2009). Yellow, high relative expression; blue, low relative expression.

(B) Ingenuity Pathway Analysis of RNA-seq data, showing *C. albicans*-exposed OKF6/TERT2 cells (untreated relative to IL-17+TNFα treated) compared to murine OPC (*C. albicans*-infected WT tongue relative to uninfected). Data indicate *Z* scores of predicted regulation by infection. Red, predicted activation; blue, predicted repression; white, no predicted regulation.

IL-17RA Expressed by OECs Contributes to Protection from Oral Candidiasis

To determine the extent to which IL-17RA expression in oral epithelium is required for immunity to OPC, $K13^{Cre}$ mice were crossed to recently developed *Il17ra*$^{fl/fl}$ mice (Kumar et al., 2016) (*Il17ra*$^{fl/fl}$$K13^{Cre+}$, also termed *Il17ra*$^{\Delta K13}$). To assess efficiency of receptor deletion, we evaluated IL-17RA in the oral cavity by immunohistochemistry (IHC). Tongue from *Il17ra*$^{fl/fl}$$K13^{Cre-}$ littermates (also termed WT), but not *Il17ra*$^{-/-}$ controls, stained positively for IL-17RA throughout the tissue (Figure 4A). In *Il17ra*$^{\Delta K13}$ mice, IL-17RA was effectively deleted in the BLs of the oral epithelium, but not other areas of the tissue (Figure 4A). To assess the consequence of OEC-specific deletion, *Il17ra*$^{\Delta K13}$ and controls were subjected to OPC, and fungal loads were assessed over 14 days. All infected animals had high *C. albicans* fungal burdens for the first 2 days post-infection. *Il17ra*$^{-/-}$ mice maintained high fungal loads through day 14. *Il17ra*$^{\Delta K13}$ mice showed a similar susceptibility profile as *Il17ra*$^{-/-}$ mice. Specifically, fungal counts on days 3–14 were statistically indistinguishable from *Il17ra*$^{-/-}$ mice (Figure 4B). Both *Il17ra*$^{\Delta K13}$ and *Il17ra*$^{-/-}$ mice lost weight progressively, whereas *Il17ra*$^{fl/fl}$$K13^{Cre-}$ littermates returned to their starting weights (Figure 4B).

The composition of saliva also influences oral *C. albicans* colonization. We previously found that saliva from *Il17ra*$^{-/-}$ mice exhibits a modestly impaired ability to kill *C. albicans* in vitro (Conti et al., 2009). AMPs in saliva, such as defensins, are derived from salivary glands but are also shed from the oral epithelium. To determine whether IL-17RA-dependent factors from OECs contributed to salivary candidacidal function, mice were administered carbachol to induce salivation, saliva was incubated with 10^6 *C. albicans* cells for 1 hr, and cultures plated for colony enumeration. Saliva from sham-infected mice exhibited similar baseline candidacidal activities (Figure 4C). After infection, saliva from *Il17ra*$^{\Delta K13}$ mice and *Il17ra*$^{fl/fl}$$K13^{Cre-}$ controls maintained similar levels of *Candida*-killing capacity, while *Il17ra*$^{-/-}$ saliva showed a trend to reduced killing efficiency. Consequently, the increased susceptibility of *Il17ra*$^{\Delta K13}$ mice to OPC does not appear to be due to defects in salivary function.

OEC-Specific IL-17RA Regulation of Neutrophils

IL-17RA induces expression of neutrophil-related chemokines and growth factors during OPC, such as CXC chemokines and G-CSF (Conti et al., 2009; Huppler et al., 2014). Here, we evaluated neutrophil recruitment to the supra-and sub-basal layers of epithelial tissue. Immediately after infection (day 1), there was significantly reduced neutrophil infiltration to the suprabasal region in *Il17ra*$^{-/-}$ mice compared to *Il17ra*$^{fl/fl}$$K13^{Cre-}$ (WT) controls. Surprisingly, however, there was no difference in neutrophil numbers in *Il17ra*$^{\Delta K13}$ mice in this area compared to WT (Figures 5A and 5B). At later times (days 3–5), neutrophil numbers were elevated in both *Il17ra*$^{-/-}$ and *Il17ra*$^{\Delta K13}$ suprabasal tissue compared to WT, which probably reflects the ongoing *C. albicans* infection in knockout, but not WT, mice (Figures 5A and 5B). In sub-basal layers, there was no difference in neutrophil counts in any of the mice (Figures 5A and 5B). However, although neutrophil levels are elevated, they never reach the same frequency as in WT mice. Therefore, OEC-specific deletion of IL-17RA does not cause impaired local recruitment of neutrophils to the superficial epithelial layer, though a complete IL-17RA deficiency does (Conti et al., 2009). As an additional assessment of pathology, we quantified tissue hyperplasia, measured as the distance between the outermost keratinized portion of the epithelium and stem cells of the basal layer. In line with the sustained levels of neutrophils in the *Il17ra*$^{\Delta K13}$ mice, the suprabasal epithelium was hyperplastic compared to *Il17ra*$^{fl/fl}$$K13^{Cre-}$ controls (Figures 5C and 5D). This finding indicates that IL-17RA signaling in OECs is not required for cellular proliferation.

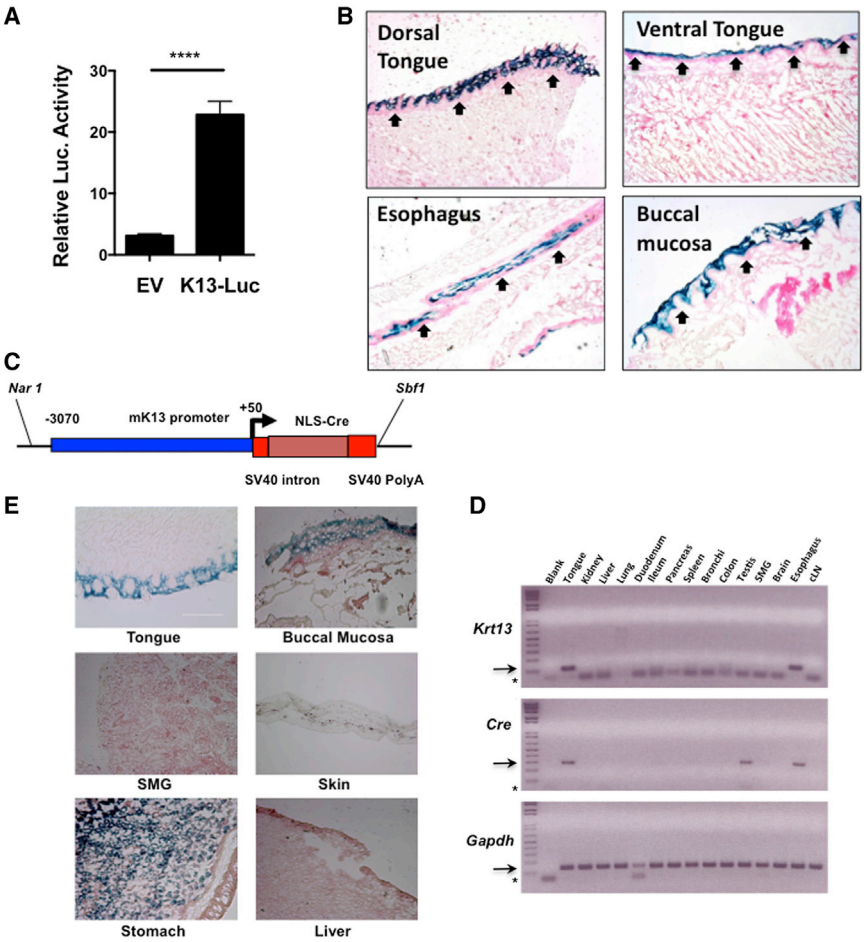

Figure 3. The K13 Promoter Shows Specific Activity in Oral Epithelium

(A) The K13 promoter in the pGL3-Luc vector was transfected into oral epithelial IMOK cells, and Luc activity was assessed after 24 hr. Data show mean + SEM. ***$p < 0.01$ by Student's t test. EV, empty vector.

(B) $K13^{LacZ}$ mice were analyzed for β-galactosidase activity by Xgal staining.

(C) K13-Cre cassette used to create $K13^{CRE}$ mice.

(D) Indicated tissues from $K13^{CRE}$ founder #176 were analyzed for *Krt13* and *Cre* by qPCR. SMG, submandibular salivary gland; cLN, cervical lymph node. Arrow, specific PCR product. *Unincorporated PCR primers.

(E) Tissues from $K13^{CRE}$-*Rosa*26^{LacZ} mice were stained with Xgal to assess β-galactosidase activity. Data are from two to four independent experiments.

We next asked whether the neutrophils migrating to the superficial epithelium functioned normally by staining tissue for myeloperoxidase (MPO). Neutrophils from all mice stained positive for MPO, indicating that the neutrophils within the tissue appear functional (Figure 5E). Collectively, these data confirm and extend our previous findings that IL-17RA regulates neutrophil migration, but indicate, surprisingly, that the neutrophil defect is less marked in *Il17ra*$^{\Delta K13}$ mice compared to *Il17ra*$^{-/-}$ mice. Accordingly, the high susceptibility of *Il17ra*$^{\Delta K13}$ mice to OPC is likely not due to neutrophil defects.

OPC-Induced Gene Profiles in Il17ra$^{\Delta K13}$ Mice: Role for BD3

To gain an unbiased assessment of genes regulated by IL-17RA within OECs, we performed RNA-seq analysis of tongue tissue from *Il17ra*$^{fl/fl}$K13^{Cre-}, *Il17ra*$^{\Delta K13}$ and *Il17ra*$^{-/-}$ mice 1 day after oral *C. albicans* infection (see Figure 2). A panel of genes emerged that were expressed in *Il17ra*$^{fl/fl}$K13^{Cre-} littermate controls (WT) upon infection (i.e., that were different from sham-infected mice) that were differentially regulated in both *Il17ra*$^{\Delta K13}$ and *Il17ra*$^{-/-}$ mice after infection (Figures 6A and S1). Notably, the majority of the gene changes that are absent in the *Il17ra*$^{-/-}$ mice are also absent in *Il17ra*$^{\Delta K13}$ mice. These overlapping genes included known IL-17RA targets important in antifungal defense, including AMPs (*Defb3* and *S100A9*) and chemokines (*Cxcl1* and *Cxcl5*) (Tables S1 and S2). These data confirmed that the OEC-specific deletion of IL-17RA is concordant with the full knockout with respect to early antifungal responses. However, some genes are also regulated by other IL-17RA-expressing tissues.

To understand the kinetics of the OEC-dependent gene expression, we investigated the profile of a subset of genes identified by RNA-seq over a 5 day time course. We first focused on neutrophil-associated genes including *S100A9*, chemokines (*Cxcl1*, also known as KC; *Cxcl2*; and *Cxcl5*), and G-CSF (*Csf3*). Although all these genes were reduced in *Il17ra*$^{\Delta K13}$ mice on day 1 (Figures 6A and 6B), expression recovered by days 2–3, consistent with the elevated levels of neutrophils observed in these mice compared to the *Il17ra*$^{-/-}$ mice (Figure 5). WT mice also showed reduced expression of these genes, presumably because they have cleared the infection and the overall immune response has returned to baseline. Lipocalin-2 (*Lcn2*), while not required for the response to OPC (Ferreira et al., 2014), serves as a sensitive measure of IL-17 signaling activity in OPC and other settings (Shen et al., 2006). There was impaired expression of *Lcn2* in both *Il17ra*$^{\Delta K13}$ mice and *Il17ra*$^{-/-}$ mice at day 1, the only time point when this gene was high in WT mice (Figures 6A and 6B).

BDs exhibit potent anti-*Candida* activity. *Defb1* was impaired at day 1 in *Il17ra*$^{-/-}$ mice, consistent with its known role in immunity to OPC (Tomalka et al., 2015), and here we demonstrate an IL-17-dependent activation of this factor. However, *Defb1* was not altered in *Il17ra*$^{\Delta K13}$ mice. *Defb2* and *Defb4* were not altered at any time points (data not shown). In contrast, *Defb3* expression correlated well with OPC susceptibility in *Il17ra*$^{\Delta K13}$ mice, for its expression was markedly impaired at days 1–3 in *Il17ra*$^{\Delta K13}$ and *Il17ra*$^{-/-}$ mice compared to WT (Figures 6A and 6B). To determine whether BD3 protein was reduced in situ, we performed IHC with anti-BD3 Abs on *Il17ra*$^{fl/fl}$K13^{Cre-}, *Il17ra*$^{\Delta K13}$, and *Il17ra*$^{-/-}$ tongue sections after OPC (Figure 6C).

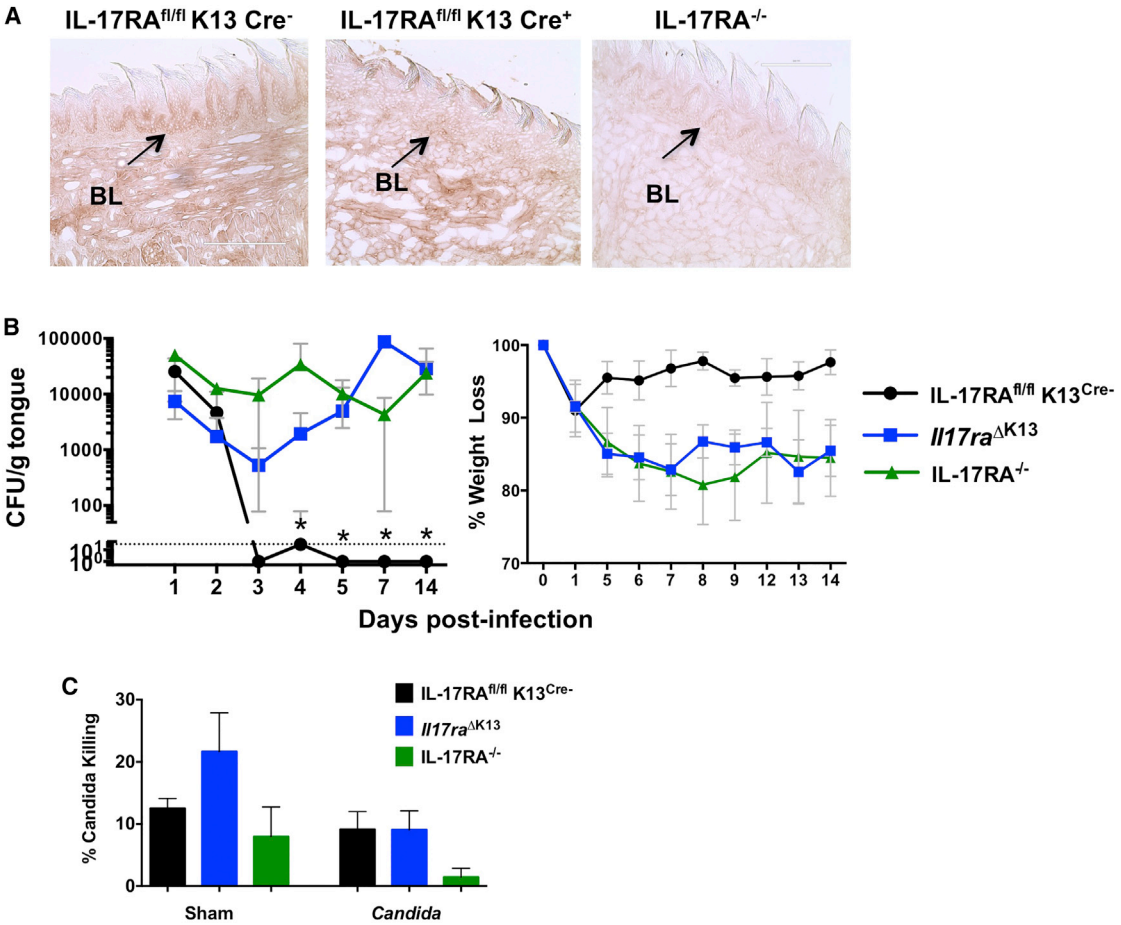

Figure 4. IL-17RA in OECs Is Necessary for Immunity to OPC

(A) Expression of IL-17RA was assessed by IHC. BL, suprabasal layer (n = 3).

(B) Indicated mice were subjected to OPC as in Figure 1. Left: mean fungal loads + SD at the indicated days post infection (left). *Il17ra*$^{-/-}$ (green, n = 3–6), Cre^{+} (blue, n = 3–7), and Cre^{-} (black, n = 3–4). Right: mean percent weight change relative to day 0 + SD. Data are from two to four experiments per time point.

(C) Saliva was incubated with *C. albicans* cells for 1 hr at 37°C and CFU was assessed by plating. Data show mean + SEM. Experiment was done twice.

BD3 was expressed in the superficial tongue and the OECs in *Il17ra*$^{fl/fl}$K13^{Cre-} mice, but was absent in both the *Il17ra*$^{\Delta K13}$ and *Il17ra*$^{-/-}$ OECs. We next confirmed that human OECs stimulated with IL-17 induced secretion of BD2, the human ortholog of murine BD3 (Figure 6D). Since clinical blockade of IL-17 is associated with impaired epithelial repair in the gut (Whibley and Gaffen, 2015), we asked whether IL-17 can protect from cell damage induced by *C. albicans*. However, IL-17 did not alter the cytotoxicity arising from *C. albicans* infection of cultured OECs, measured by secretion of lactate dehydrogenase (LDH) (Figure 6E). Finally, mice lacking BD3 (*Defb3*$^{-/-}$) were just as susceptible to OPC as *Il17ra*$^{-/-}$ mice (Figure 6F). Thus, OEC-derived BD3 appears to account for the high susceptibility of *Il17ra*$^{\Delta K13}$ mice to OPC.

DISCUSSION

Our understanding of fungal pathogens has historically lagged behind that of bacteria or viruses (Brown et al., 2012), but recent discoveries in studies of *C. albicans* have dramatically advanced the field. These insights include (1) the identification of C-type lectin receptors as key pattern recognition receptors for fungi and (2) the recognition that IL-17 signaling is central to antifungal immunity (Hernández-Santos and Gaffen, 2012). Mice lacking IL-17RA or IL-17RC are susceptible to OPC and dermal candidiasis (Conti et al., 2009, 2015; Ho et al., 2010; Kagami et al., 2010). This finding was upheld in humans with IL-17R mutations (Ling et al., 2015; Puel et al., 2011). Our bioinformatics data provide additional evidence that the murine response to oral *C. albicans* is concordant with human OECs (Figure 2). Not only are there conspicuous similarities in individual genes or their orthologs (BD2/3, chemokines, lipocalin-2; Jia et al., 2000), but at a global level, the pathways induced by *C. albicans* show strong parallels.

Nonetheless, it has not been obvious in which tissue(s) or cell types IL-17 signaling is required. Mesenchymal and epithelial cell types are generally considered the main responders to this cytokine. Their sensitivity is thought to be due to high expression of IL-17RC on these cell types, whereas this subunit is expressed at low or undetectable levels in hematopoietic cells (Ishigame et al., 2009; Kuestner et al., 2007). IL-17RA is

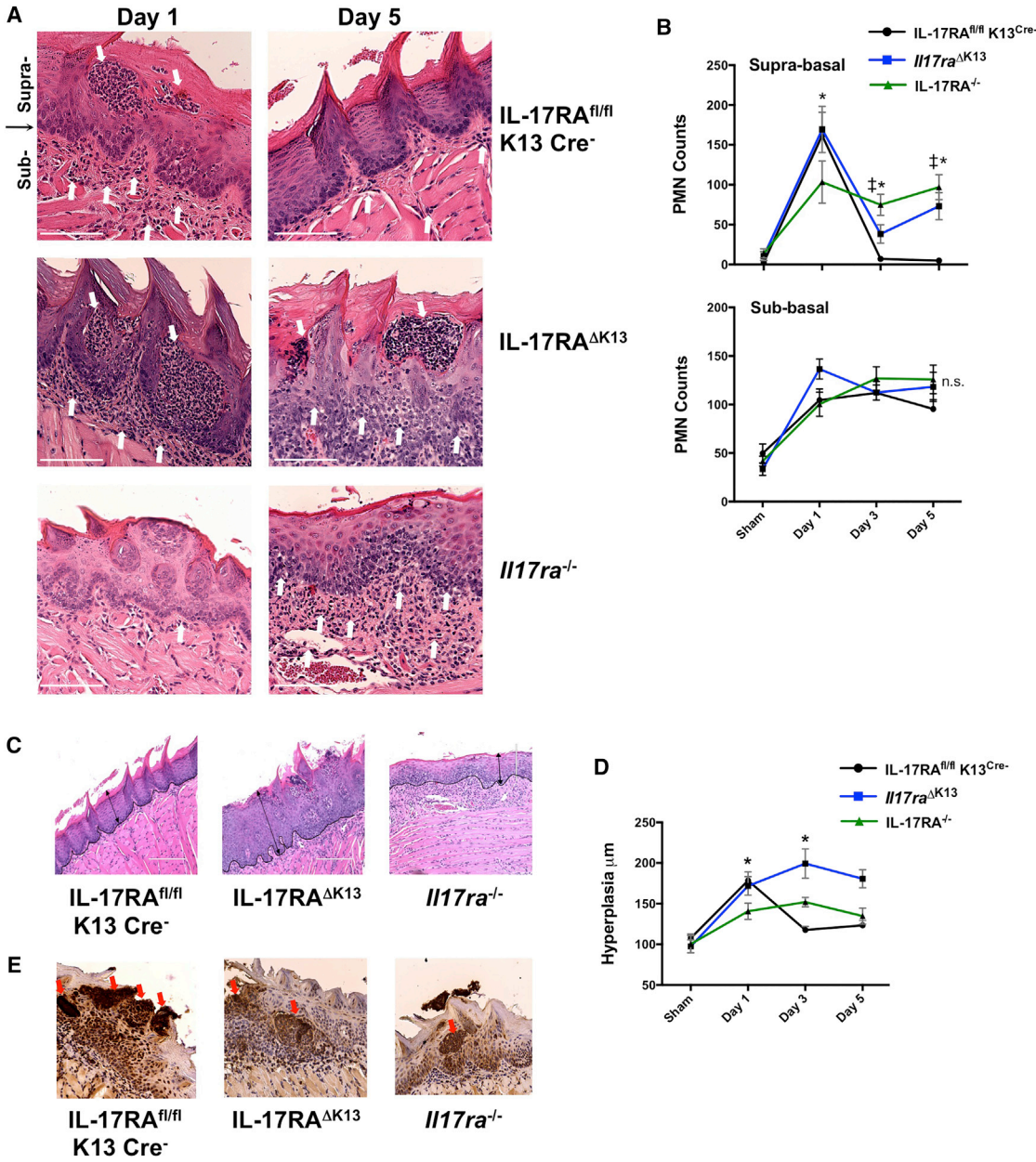

Figure 5. Tissue Changes in *Il17ra*^{K13} Compared to *Il17ra*$^{-/-}$ Mice Following *C. albicans* Infection

(A) Images (20×) from H&E-stained tongue sections from the indicated *C. albicans*-infected mice at day 1 or 5. Black arrow indicates division between supra- and sub-basal layers. White arrows indicate neutrophils. Scale bar, 100 μM.

(B) Quantification of neutrophils in H&E images (n = 4 mice/group, minimum of 6 sections per mouse). *p < 0.05, WT (Cre$^-$) versus *Il17ra*$^{-/-}$; ‡ p < 0.05, *Il17ra*^{K13} versus WT (Cre$^-$) by ANOVA with Kruskal-Wallis correction. Data indicate means + SEM.

(C) Hyperplasia is indicated as thickening of epithelium between basal cell layer to superficial keratinized layer. Arrows indicate distance measured in microns using ImageJ.

(D) Quantification of hyperplasia by blinded assessors (n = 4 mice/group, minimum 4 sections per mouse). *p < 0.05 compared to WT. ‡p < 0.05 compared to *Il17ra*$^{\Delta K13}$ by ANOVA. Data indicate means + SEM.

(E) MPO staining in infected mice. Arrows indicate areas of high MPO staining. Experiments were performed on sections from three to five mice per strain. All experiments were done twice.

expressed more broadly than IL-17RC, with particularly high levels in hematopoietic cells (Ishigame et al., 2009; Yao et al., 1995; Ye et al., 2001). Here, we found that the hematopoietic compartment was dispensable for immunity to OPC (Figure 1), similar to the tissue dependence of IL-17RA in a murine arthritis model (Lubberts et al., 2005). However, this contrasts with a report that NK cells are the essential IL-17-responsive cell type in systemic candidiasis (Bär et al., 2014).

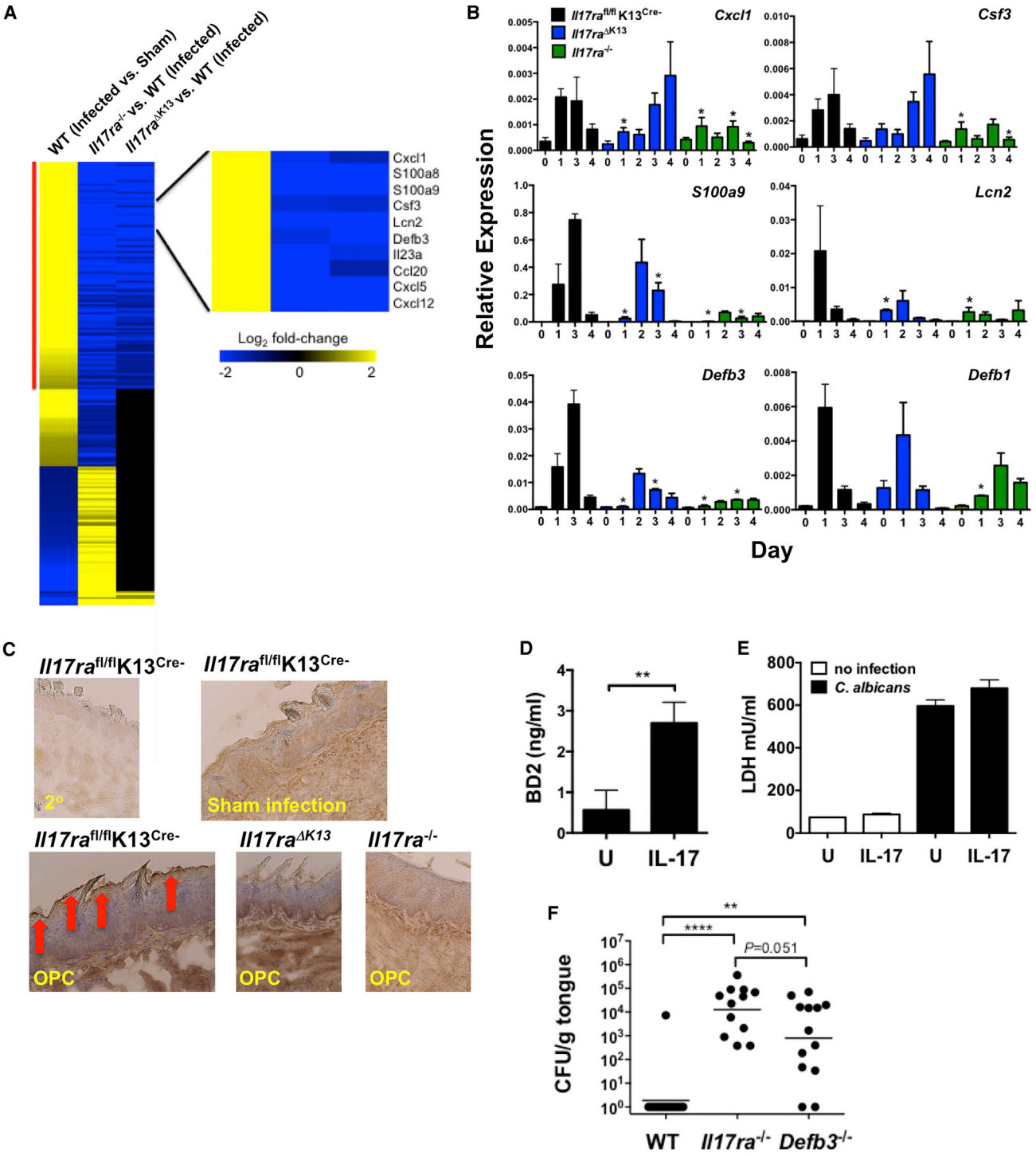

Figure 6. *C. albicans*-Induced Gene Expression Changes Require Epithelial IL-17RA and Demonstrate a Role for BD3

(A) RNA-seq was performed 24 hr after infection (n = 3). Heatmap shows genes that are differentially expressed after infection of WT mice for 24 hr and whose regulation is altered in infected *Il17ra*$^{-/-}$ or *Il17ra*^{K13} mice. Values = log (base 2) fold change. Red bar indicates genes whose *C. albicans*-induced regulation required IL-17RA in oral epithelium.

(B) Tongue RNA from *C. albicans*-infected mice was assessed by qPCR (n = 6–9). Bars indicate mean + SEM. *p < 0.05 by ANOVA and Student's t test.

(C) Tongue sections were stained with α-BD3 Abs or 2° Ab. Arrows indicate suprabasal areas (n = 3).

(D) TR146 cells were incubated with IL-17 for 20 hr ± IL-17A. BD2 in supernatants was assessed by ELISA. Bars indicate mean + SEM. **p < 0.01.

(E) TR146 cells were infected with *C. albicans* yeast at an MOI of 0.01 ± IL-17A (200 ng/mL) for 24 hr. Secreted LDH was assessed in triplicate.

(F) Indicated mice were subjected to OPC and oral fungal loads assessed after 5 days. Bars indicate geometric mean. **p < 0.01. ****p < 0.0001. All experiments were done at least twice. See also Figure S1 and Table S1.

Although OECs comprise only a fraction of total tongue tissue, they are the first cells to encounter *C. albicans* in the mouth. Until now, genetic tools to study oral tissue have been limited. Epithelial tissues are classified by differentiation-specific expression of keratins, cytoskeletal proteins that provide structural integrity and function as a protective barrier. In oral and buccal epithelia, K13 and K4 are expressed in superficial and intermediate tissue layers (Presland and Dale, 2000). We show that mice expressing Cre under control of the K13 promoter exhibit efficient and specific conditional deletion within these tissues (Figures 2 and 3). The creation of this $K13^{CRE}$ line will be valuable for many areas of inquiry, such as other oral/esophageal/vaginal infections, oral cancer, and wound healing in non-keratinized tissues.

There is an intriguing concordance between K13 expression and sites of *C. albicans* infections, including oral and esophageal mucosa and vaginal epithelium (data not shown). To date, the participation of IL-17 in vaginal candidiasis remains controversial, but this mouse system will be useful for probing the role of IL-17 in this tissue (Ibrahim et al., 2013; Pietrella et al., 2011; Yano et al., 2012). It is unlikely that K13 acts as a receptor for *C. albicans*, though this possibility cannot be formally excluded. Rather, epithelial cells within non-keratinized layers have a rapid turnover (~4 days), compared to ~2 weeks in skin. These sites are also thinner and more permeable than skin, and thus may be more amenable to fungal invasion. Recent data showed that a *C. albicans*-derived toxin termed Candidalysin is required to establish pathogenic infections of the oral mucosa (Moyes et al., 2016). Conceivably, the cellular receptor for Candidalysin is more highly expressed in $Krt13^{+}$ OECs.

Our data with $Il17ra^{\Delta K13}$ mice suggested that the bulk of IL-17RA-driven immunity in the context of OPC is from OECs (Figure 4). While there was not a statistically significant difference in fungal burdens between $Il17ra^{\Delta K13}$ and $Il17ra^{-/-}$ mice, there was a consistent trend to lower fungal counts in the $Il17ra^{\Delta K13}$ animals, suggesting there may be contributions of IL-17RA in other cells. This idea is supported by the global analysis of the gene differences between $Il17ra^{\Delta K13}$ and $Il17ra^{-/-}$ mice during infection. Although expression of many genes was conserved during OPC on day 1, there were notable differences (Figures S1 and 6B). Genes associated with neutrophils are a case in point; many of the factors that regulate neutrophil chemotaxis (*Cxcl1*), expansion (*Csf3*), or function (*S100A9*) were more severely impaired in the total knockout compared to the OEC-specific deletion. These data are consistent with histological analyses, which demonstrated reduced recruitment of neutrophils to $Il17ra^{-/-}$ suprabasal oral tissue compared to comparable sites in $Il17ra^{\Delta K13}$ mice (Figure 5). In contrast, expression of *Defb3* was markedly reduced in $Il17ra^{\Delta K13}$ mice, correlating well with fungal loads (Figure 6). These data collectively imply that IL-17RA-driven expression of neutrophil-attractive factors is driven by OECs at early time points (day 1) but by other cells at subsequent times (days 3–5).

The nature of other IL-17RA-responsive cells is an intriguing question. We speculate that mesenchymal cells (e.g., muscle or fibroblastic cells) within the oral mucosa may be involved. We base this hypothesis on a study in a murine psoriasis model that showed a similar dichotomy in IL-17-dependent functions by cell type. In this model, a deficiency in Act1 (a key adaptor downstream of IL-17RA) in dermal keratinocytes impairs neutrophilic abscess formation, whereas Act1 deleted only in dermal fibroblasts impacts keratinocyte hyperproliferation (Ha et al., 2014). Notably, mice or humans lacking Act1 are also susceptible to OPC (Boisson et al., 2013; Ferreira et al., 2014). Although we did not see an obvious contribution of saliva in the $Il17ra^{\Delta K13}$ mice (Figure 4), this assay is not very sensitive, so we cannot rule out a role for IL-17RA in salivary glands.

IL-17 regulates expression of BDs at mucosal surfaces (Aujla et al., 2007; Kao et al., 2004). IL-17 is implicated in regulation of murine BD3 and its human homolog BD2 (Figures 1, 5, and 6; Conti et al., 2009; Jia et al., 2000; Simpson-Abelson et al., 2015). The importance of BD3 was confirmed by the observation that $Defb3^{-/-}$ mice showed the same susceptibility to OPC as $Il17ra^{-/-}$ animals. BD3 exerts direct antifungal activity on *C. albicans* (Edgerton et al., 2000; Joly et al., 2004). Additionally, BD3 binds to CCR6, potentially allowing it to serve as a chemoattractant for IL-17-expressing cells that express this receptor (Yamazaki et al., 2008). CCL20 is another ligand for CCR6 that is induced during OPC by IL-17RA signaling (Figure 6). Intriguingly, CCL20 also exhibits candidacidal activity (Yang et al., 2003). BD1 is implicated in OPC (Tomalka et al., 2015), and our data indicate this is also IL-17RA dependent. Thus, genes expressed in OPC have the capacity to function as AMPs and chemoattractants, establishing a feedforward amplification loop that controls fungal infections.

Most data indicate that IL-17A is the dominant cytokine in OPC, but other type 17 cytokines also contribute. Patients with autoimmune polyendocrinopathy syndrome (*AIRE* deficiency) produce neutralizing Abs against IL-17A, IL-17F, and IL-22, correlating with CMC (Kisand et al., 2010; Puel et al., 2010). $Il17rc^{-/-}$ mice (which respond only to IL-17A and IL-17F) phenocopied $Il17ra^{-/-}$ mice (Ho et al., 2010), later confirmed in humans (Ling et al., 2015). $Il17a^{-/-}$, but not $Il17f^{-/-}$, mice are susceptible to OPC, but dual blockade of IL-17A and IL-17F increases susceptibility over anti-IL-17A treatment alone (Gladiator et al., 2013; Whibley et al., 2016). Th17 cells also make IL-22, and $Il22^{-/-}$ mice are moderately susceptible to OPC (Conti et al., 2009). IL-25 (IL-17E) has not been evaluated in OPC, to our knowledge, but there is no change in *Il17e* expression in mice upon oral infection with *C. albicans* (Whibley et al., 2016). Thus, there appears to be cooperative activity of type 17 cytokines in immunity to oral *C. albicans* infections.

In summary, this and the accompanying report by Chen et al. (2016) provide insight into the tissue-selective role by which IL-17R-dependent immunity to mucosal pathogens is controlled. Drugs that target IL-17- and Th17-related pathways are now approved for moderate-severe plaque psoriasis (Sanford and McKeage, 2015); inevitably, as their clinical use increases, so does the potential for opportunistic mucosal infections. Ultimately, studies like these may inform the development of better targeted therapeutics for human disease.

EXPERIMENTAL PROCEDURES

K13 Promoter

The *Krt13* promoter (−3,090 to +40) was obtained by PCR from bacterial artificial clone (BAC) RP23-10A2 and cloned into the pGL3-basic vector (Promega). IMOK cells (Parikh et al., 2008) were transfected with the reporter with

FuGENE 6, normalized to a pCMV-LacZ control. Function was assessed with the Luciferase Assay system (Promega), and β-galactosidase with the Galacton Plus kit (Life Technologies).

Cell Culture

OKF6/TERT2 cells (Dickson et al., 2000) were cultured in serum-free fibroblast media, 25 μg/mL bovine pituitary extract and 2 μg/mL EGF (Life Technologies). Cells were treated with TNFα (0.5 ng/mL) and IL-17A (50–200 ng/mL) (R&D Systems) for 24 hr and then infected with 1×10^7 *C. albicans* yeast for 5 hr. TR146 cells were from ECACC and cultured in DMEM-F12/15% fetal bovine serum (FBS).

Mice

Il17ra$^{-/-}$ mice and anti-IL-17RA Abs were a gift from Amgen. *Il17ra*$^{fl/fl}$ mice were made as described (Kumar et al., 2016). *Defb3*$^{-/-}$ mice were from the MMMRC (UC Davis). K13^{LacZ} transgenic mice were created with a K13-LacZ cassette at the Roswell Park Cancer Institute (RPCI) Transgenic Core Facility. Oral tissue sections were stained with Xgal to detect β-galactosidase activity. K13^{CRE} transgenic mice were created with the *Krt13*-NLS-Cre by the Pittsburgh Transgenic and Gene Targeting Core Facility, and founders identified by PCR. C57BL/6 and *Gt(Rosa)26*^{tm1Sor} mice were from The Jackson Laboratory. Experiments were performed in accordance with IACUC protocols approved by the University of Pittsburgh, the University of Toledo, the State University of New York at Buffalo, or RPCI.

Oral Candidiasis

OPC was performed by sublingual inoculation with a cotton ball saturated in *C. albicans* (CAF2-1) for 75 min (Solis and Filler, 2012). Tongue homogenates were dissociated on a GentleMACS (Miltenyi Biotec) and serial dilutions plated on YPD+Amp. 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.

Salivary Assays

Saliva was collected after carbachol injection (100 μL, 10 μg/mL) and used immediately (Conti et al., 2009). A total of 90 μL saliva was incubated with 10^4 cells of *C. albicans* for 1 hr at 37°C, plated in triplicate, and assayed after 2 days for CFU enumeration on YPD-Amp.

IHC, ELISA, and LDH Assays

Cryosections were stained with α-IL-17RA mAbs (Amgen), α-MPO mAbs (R&D Systems), or anti-rat BD3 Abs (Santa Cruz) using the BioLegend IHC Protocol for Frozen Tissue or Immunocruz LSAB Staining System. Image analysis was performed by two independent assessors in a blinded fashion. BD2 ELISAs were performed with Abs from Peprotech. LDH assays were performed with a CytoTox 96 Assay System (Promega).

RNA-Seq and qPCR

RNA-seq mouse libraries were prepared with total RNA using a Qubit 2.0 fluorometer (Thermo Fisher) and Agilent Bioanalyzer TapeStation 2200 (Agilent Technologies). For OKF6-TERT2 in vitro infections, RNA-seq libraries (non-strand specific, paired-end) were prepared with the TruSeq RNA kit (Illumina). A total of 100 nucleotides were determined from each end of cDNA fragments using the HiSeq platform. Single reads were aligned to the UCSC mouse or human reference genomes (mm10, GRCm38.75; Ensembl GRCh38) using TopHat2. Differential gene expression was assessed using DESeq (Bioconductor). Pairwise differential expression was quantified with Cuffdiff. Cufflinks was used to determine FPKM levels for each gene from the STAR alignment and was used as input for Cuffdiff. Read counts were then normalized across all samples and differentially expressed genes were determined by adjusted p value with a threshold of 0.05.

For real-time PCR, RNA was isolated from tongue with RNeasy Mini Kits (QIAGEN), and cDNA generated with Superscript III First Strand Kits (Invitrogen). Real-time qPCR using SYBR Green FastMix ROX (Quanta Biosciences) was performed using a 7300 Real-Time instrument (Applied Biosystems) and normalized to GAPDH. Primers were from Superarray Biosciences or QuantiTect Primer Assays (QIAGEN).

ACCESSION NUMBERS

Raw sequencing reads have been submitted to the Sequence Read Archive under ID code SRA: SRP075350. The RNA-seq data for the in vitro infections have been deposited under ID code SRA: SRP077728.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, one figure, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2016.10.001.

AUTHOR CONTRIBUTIONS

Conceptualization, S.L.G., H.R.C., S.S., S.G.F., and V.M.B.; Methodology, S.L.G., S.S., and J.K.K.; Formal Analysis, V.M.B.; Investigation, H.R.C., E.E.C., J.P.H., B.G.M., B.M.C., M.R.H., L.B., N.S., J.A.C., A.H.V., S.D., A.G.H., A.V.G., J.P.R., D.L.S., S.L.G., and S.S.; Resources, J.K.K. and J.R.N.; Writing – Original Draft, S.L.G., H.R.C., and V.M.B.; Writing – Review & Editing, J.K.K., J.R.N., S.G.F., and S.S.; Supervision, H.R.C. and S.L.G.; Funding Acquisition, S.L.G., H.R.C., V.M.B., S.G.F., and J.K.K.

ACKNOWLEDGMENTS

S.L.G. was supported by the NIH (AI107825, DE022550, and DE023815). H.R.C. was supported by F32-DE023293 and the University of Toledo. V.M.B. and S.G.F. were supported by U19AI110820. J.K.K. was supported by DE022550 and R37-HL079142. A.G.H. was supported by DE018279 and a Department of Veterans Affairs Merit Award I01BX002607. J.R.N. was supported by the Medical Research Council (MR/M011372/1) and Biotechnology & Biological Sciences Research Council (BB/N014677/1). This work is solely the responsibility of the authors and does not necessarily reflect the views of the NIH or other funding bodies. We thank W. Horne for assistance with RNA-seq. We thank Amgen for *Il17ra*$^{-/-}$ mice and anti-IL-17RA Abs, and M.J. McGeachy and P.S. Biswas for helpful suggestions and critical reading. S.L.G. has received research grants from Novartis and Janssen, and is on the Scientific Advisory Board of Lycera. J.K.K. has received consultant fees and travel reimbursements from Boeringher-Ingelheim.

Received: March 20, 2016
Revised: August 16, 2016
Accepted: September 21, 2016
Published: October 27, 2016

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