IL-17-mediated antifungal defense in the oral mucosa is independent of neutrophils

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Interleukin-17 (IL-17)-mediated immunity has emerged as a crucial host defense mechanism against *Candida albicans* infections in mucosal tissues and the skin. The precise mechanism by which the IL-17 pathway prevents fungal outgrowth has not been clarified. Neutrophils are critical for limiting fungal dissemination and IL-17 is generally thought to act by regulating neutrophil mobilization and trafficking to the site of infection. Using a mouse model of oropharyngeal candidiasis (OPC), we found that strikingly the IL-17 pathway is not required for the neutrophil response to *C. albicans*. Mice deficient for the IL-17 receptor subunits IL-17 receptor A (IL-17RA) or IL-17RC or mice depleted of IL-17A and IL-17F exhibited a normal granulocyte colony-stimulating factor (G-CSF) and CXC-chemokine response and displayed no defect in neutrophil recruitment or function. Instead, the inability of these mice to clear the fungus was associated with a selective defect in the induction of antimicrobial peptides (AMPs) in the epithelium that resulted in persistent fungal colonization. Importantly, this antifungal mechanism of IL-17A and IL-17F did not extend to the closely related family member IL-17C. Together, these data uncouple IL-17-dependent effector mechanisms from the neutrophil response and reveal a compartmentalization of the antifungal defense in the oral mucosa providing a new understanding of IL-17-mediated mucosal immunity against *C. albicans*.

INTRODUCTION

Interleukin-17 (IL-17)-mediated immunity has emerged as a key mechanism for protection from mucosal fungal infections. Genetic defects in the IL-17 pathway are associated with chronic mucocutaneous candidiasis (CMC), a condition characterized by recurrent or persistent infections of the skin, nails, genital, and oral mucosa with *Candida* species.¹ The first direct evidence that IL-17 is important in CMC protection was the identification of patients with complete autosomal-recessive deficiency in the IL-17 receptor A (IL-17RA) or partial autosomal-dominant IL-17F deficiency.² More recently, patients with missense mutations in the adaptor molecule Act1³ have been identified. In addition, patients with loss-of-function mutations in *STAT3* or gain-of-function mutations in *STAT1*, which exhibit a deficit in IL-17-producing circulating T cells, and patients with mutations in *AIRE* with high titers of neutralizing autoantibodies to IL-17A, IL-17F, and IL-22 display CMC.^{4–8} Together, these findings indicate that IL-17 cytokines have a non-redundant role in antifungal defense in barrier tissues. Experiments in mice have reproduced the strict

dependence of mucocutaneous defense against *C. albicans* on IL-17 signaling.^{9,10} The family of IL-17 cytokines consists of six members (IL-17A–IL-17F). The most well-studied cytokines IL-17A and IL-17F, which can form homodimers and heterodimers, signal via the heterodimeric receptor complex IL-17RA–IL-17RC, while the related cytokine IL-17C uses a dimeric receptor composed of IL-17RA and IL-17RE for signaling.¹¹ T helper cells are considered the major producers of IL-17A and IL-17F.¹² They are thought to contribute to long-term protection from fungi, in particular from *C. albicans*, which is part of the normal commensal flora in Man.¹³ Consistent with this notion, *C. albicans*-specific memory T cells in humans belong preferentially to the IL-17-secreting subset,¹⁴ and antigen-specific T helper-17 cells promote protection from fungal challenge when induced in an experimental vaccination setting.^{15–17} In addition to CD4⁺ T cells, IL-17 can be secreted by a wide variety of cells of the innate immune system,¹⁸ which are particularly relevant during acute infection. Accordingly, IL-17A- and IL-17F-producing innate lymphoid cells (ILCs) have a key role in mediating IL-17-dependent protection

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against *C. albicans* in a mouse model of oropharyngeal infection.¹⁹

Although IL-17 cytokines have been studied widely in recent years, their mode of action remains poorly understood. While IL-17RA is expressed broadly, on hematopoietic and non-hematopoietic cells, the co-receptor IL-17RC has a more restricted expression pattern.²⁰ Non-hematopoietic cells including epithelial cells, endothelial cells, and fibroblasts are thought to be the most prominent target cells of IL-17A and IL-17F. Ligand binding to the IL-17RA-IL-17RC heterodimer activates nuclear factor kappa-B via the adaptor protein Act1 and tumor necrosis factor receptor-associated factor 6 as well as the CCAAT/enhancer-binding proteins C/EBP- β and C/EBP- δ to promote transcription and increase mRNA stability of target genes. Early studies on the biological function of IL-17A linked it to neutrophil biology. They demonstrated that IL-17 induces the production of granulocyte colony-stimulating factor (G-CSF), IL-6, and the neutrophil chemotactic factor IL-8 in human cells,^{21,22} and that forced expression of IL-17 via different mechanisms (i.e., induction of endogenous IL-17 by endotoxin, overexpression of adenovirus-encoded IL-17A, or recombinant IL-17A administration) results in a massive neutrophilia.^{22–24} Disruption of the IL-17 pathway has been reported to cause a defective neutrophil response in several experimental settings. For example, pulmonary infection with *Klebsiella pneumonia* and *Staphylococcus aureus* in IL-17RA-deficient mice causes a reduced induction of G-CSF, granulocyte macrophage colony-stimulating factor, and neutrophil-attracting chemokines^{25,26} and is associated with decreased neutrophil recruitment to the site of infection and impaired bacterial control.^{26,27} Likewise, IL-17 is also involved in the neutrophil-mediated control of *S. aureus* infection in the skin.²⁸ Chemokine and cytokine induction and neutrophil recruitment is also affected in IL-17-deficient mice in response to *Mycobacterium bovis* bacille Calmette-Guérin infection associated with reduced granuloma formation.²⁹ Moreover, IL-17RA deficiency abrogates protection from the parasite *Trypanosoma cruzi* due to reduced parasite killing by neutrophils and amelioration of neutrophil-driven immunopathology.³⁰ Finally, IL-17-dependent vaccine immunity to *Blastomyces dermatitidis* correlates with increased neutrophil numbers and antifungal activity at the site of infection.¹⁷ In addition to these situations, in which the IL-17-mediated neutrophil response promotes pathogen clearance and is thus beneficial to the host, IL-17 may also have pathogenic effects when dysregulated, by mediating chronic inflammation linked to overt neutrophilia. This is for instance reported in cystic fibrosis, psoriasis, pulmonary aspergillosis or respiratory syncytial virus, and influenza virus infection.^{31–35} Together, this suggests a key role of neutrophils in IL-17-mediated responses. Surprisingly, the regulation of the neutrophil response to *C. albicans* has not been analyzed in detail despite the prominent role of IL-17 in protection from this fungus.¹

Here we show that the protective effects of IL-17 against oropharyngeal candidiasis (OPC) are independent of neutrophil effector functions. Although neutrophils are involved in

restricting the invading fungus at the site of infection, neutrophil recruitment and activity is not impaired in IL-17RA- and IL-17RC-deficient mice or in mice depleted of IL-17A and IL-17F cytokines. Instead, IL-17 signaling appears to mediate mucosal immunity against *C. albicans* by regulating the expression of antimicrobial peptides (AMPs) in the oral epithelium, which eliminate the fungus from the oral mucosa and prevent persistent infection.

RESULTS

Neutrophils are essential for fungal control in OPC

To evaluate the neutrophil response during oropharyngeal infection with *C. albicans*, we assessed the dynamics of neutrophil recruitment to the site of infection. We observed cellular infiltrates in the tongue starting as early as 16 h post infection (**Figure 1a,b** and data not shown). Neutrophils accumulated in the epithelial layer, predominantly at areas where *C. albicans* hyphae invaded the keratinocyte barrier on the dorsal and ventral side of the tongue. Fungal staining was detected within the cellular infiltrates, although the hyphae appeared degraded compared with the more peripheral long hyphae in the stratum corneum. We did not observe *C. albicans* penetrating the basement membrane and the underlying tissue. The population of infiltrating leukocytes, which consisted mainly of neutrophils with a lesser contribution of monocytes, reached maximal levels at 24–48 h post infection and declined thereafter, concomitant with resolution of infection and recovery of the mice (**Figure 1a** and data not shown). Depletion of neutrophils in wild-type mice using the NimpR14 antibody resulted in a strong impairment to control the fungus and the mice had to be killed for humane reasons at day 3 post infection (**Figure 1c** and data not shown). We next aimed to show the indispensable role of neutrophils in the model by using alternative neutrophil depletion strategies. We first used the anti-Ly6G antibody 1A8, but despite complete removal of neutrophils from the blood, large numbers of neutrophils could still be found in the tongue of infected mice, even when high doses of the antibody were administered daily (**Supplementary Figure S1A** online). Although the population of Ly6G^{hi} cells appeared to be strongly reduced in the tongue of 1A8-treated mice, careful analysis revealed that the large population of CD11b⁺Ly6C⁺ cells were in fact neutrophils coated with 1A8, which were unable to bind any additional fluorescently labelled 1A8 antibody used for the analysis (**Supplementary Figure S1A** and data not shown). Thus, we employed a combined strategy by depleting circulating neutrophils with anti-Ly6G antibody and blocking the mobilization of newly generated neutrophils from the bone marrow with anti-G-CSF antibody, respectively, as previously described.³⁶ Indeed, this led to a >95% reduction of neutrophils but no defect of inflammatory monocyte accumulation in the infected tongue (**Supplementary Figure S1B** and **C**). Similar to the NimpR14-treatment, this regimen resulted in high fungal loads 3 days post infection, a time point when the fungus was cleared in wild-type animals not given neutrophil-depleting antibodies (**Figure 1d**).

Figure 1 Neutrophils are essential for fungal control in oropharyngeal candidiasis (OPC). **(a)** CD11b⁺ Ly6G⁺ Ly6C⁺ neutrophils in the tongue of infected wild-type (WT) mice were counted by flow cytometry at the indicated time points. Data are the mean $\pm$ s.d. of 2–7 mice pooled from three independent experiments. **(b)** Sagittal sections of the tongue of infected WT mice were stained with periodic-acid Schiff (PAS) and counterstained with hematoxylin. A representative section for each time point is shown. Bar = 100 μ m. **(c)** Fungal burden in the tongue of untreated or NimpR14-treated WT mice was analyzed on day 2 and day 3 post infection. **(d)** Fungal burden in the tongue of untreated or anti-Ly6G and anti-granulocyte colony-stimulating factor (G-CSF)-treated WT mice on day 3 post infection. Data in **c** and **d** are pooled from two experiments each. Each symbol represents one mouse; the mean $\pm$ s.d. is indicated. The dotted line represents the detection limit. ** $P < 0.01$. **(e–f)** Representative sagittal tongue sections from untreated or anti-Ly6G and anti-G-CSF-treated **(e)** or NimpR14-treated **(f)** WT mice on day 1 **(e)** or day 2 **(f)** post infection and stained with PAS and hematoxylin. Representative sections are shown. Bar = 100 μ m. See also **Supplementary Figure S1**.

In the absence of neutrophils, *C. albicans* hyphae grew uncontrolled and penetrated the lamina propria of the tongue as early as 1 day post infection and much more prominently on day 2 post infection (**Figure 1e,f**). Together these data highlight the essential role of neutrophils for host protection from OPC, as was recently suggested by Huppler *et al.*³⁷ In response to infection, neutrophils form a seemingly impermeable immunological fence adjacent to the epithelial barrier, which confines the fungus to the site of infection.

IL-17A and IL-17F are dispensable for neutrophil recruitment to the oral mucosa

Based on our published results that ILC-derived IL-17A and IL-17F is required for protection from OPC during the acute phase of infection,¹⁹ we wanted to confirm that the enhanced susceptibility of IL-17-deficient animals was the result of an impaired neutrophil response. For this, we counted neutrophils

in bulk tongues by flow cytometry on day 1 post infection when fungal load was comparable in wild-type and IL-17-deficient mice. Surprisingly, we did not detect a reduction in the number of neutrophils recruited to the infected tongue in mice that were depleted of IL-17A and IL-17F in comparison with control mice that did not receive IL-17 cytokine-depleting antibodies (**Figure 2a** and data not shown). Similarly, neutrophil recruitment was normal in infected mice lacking IL-17A and IL-17F due to antibody-mediated depletion of ILCs or genetic deficiency in retinoid-related orphan receptor γ t (**Supplementary Figure S2A** and **B**). Moreover, mice with a genetic defect in the IL-17A- and IL-17F-specific receptor subunit IL-17RC did also not have defective neutrophil trafficking during OPC (**Figure 2b**). Furthermore, we did not observe any alterations in the dynamics of infiltration or in the distribution of neutrophils within the tongue in mice lacking a functional IL-17 pathway (**Figure 2c** and data not

Figure 2 The interleukin-17 (IL-17) pathway is dispensable for neutrophil recruitment to the oral mucosa. **(a)** CD11b⁺ Ly6G⁺ Ly6C⁺ neutrophils in the tongue of naive and infected wild-type (WT) mice that were treated with anti-IL-17A and anti-IL-17F or left untreated, as indicated, were counted by flow cytometry on day 1 post infection. **(b)** CD11b⁺ Ly6G⁺ Ly6C⁺ neutrophils in the tongue of naive and infected WT and *Il17rc*^{-/-} mice were counted by flow cytometry on day 1 post infection. **(c)** Sagittal sections of the tongue of WT and *Il17rc*^{-/-} mice were stained with periodic-acid Schiff (PAS) and counterstained with hematoxylin on day 1 post infection. Representative sections are shown. Bar = 100 μ m. **(d)** *Il17c* mRNA induction in the tongue of WT and *Rorc*^{-/-} mice on day 1 post infection. **(e)** Fungal burden in the tongue of WT and *Il17re*^{-/-} mice on day 7 post infection. The dotted line represents the detection limit. **(f)** Body weight of WT and *Il17re*^{-/-} mice in response to infection. **(g)** CD11b⁺ Ly6G⁺ Ly6C⁺ neutrophils in the tongue of naive and infected WT, *Il17ra*^{-/-}, and *Il17re*^{-/-} mice were counted by flow cytometry on day 1 post infection. **(h)** Granulocyte colony-stimulating factor (G-CSF) levels were quantified on day 1 post infection in the serum of naive and infected WT mice that were treated with anti-IL-17A and anti-IL-17F or left untreated (left), and in naive and infected *Il17ra*^{-/-} and *Il17re*^{-/-} mice (middle and right). In **a**, **b**, **d**, **e**, **g**, and **h**, each symbol represents one mouse; the mean $\pm$ s.d. is indicated. In **f**, data shown are then mean $\pm$ s.d. of six mice. Data are pooled from at least two independent experiments in **a**, **b**, **f**, **g**, and **h**. * P < 0.05; ** P < 0.01; *** P < 0.001. Differences between infected groups are not statistically significant in any of the panels. See also **Supplementary Figure S2**.

shown). Following from these unexpected results, we questioned whether other pro-inflammatory IL-17 cytokines may compensate for the absence of IL-17A and IL-17F or IL-17RC. We found that IL-17C was strongly induced by *C. albicans* (**Figure 2d**). Consistent with published data,^{38,39} IL-17C was produced by CD45-negative cells in the oral mucosa and independently of the transcription factor retinoid-related orphan receptor γ t (**Figure 2d** and data not shown). However, *Il17re*^{-/-} mice lacking the IL-17C-specific receptor subunit displayed normal numbers of neutrophils infiltrating the tongue on day 1 post infection and were as resistant to OPC as wild-type animals. They cleared the fungus, recovered their normal weight, and showed no sign of pathology by day 7 post infection (**Figure 2e–g**). Together, these data suggested that (pro-inflammatory) IL-17 cytokines

are dispensable for promoting the neutrophil response during OPC. Redundancy between IL-17A, IL-17F, and IL-17C could be excluded by depleting IL-17A and IL-17F in *Il17re*^{-/-} mice (data not shown). Accordingly, undiminished neutrophil numbers were also enumerated in the tongue of infected *Il17ra*^{-/-} mice lacking the common IL-17 receptor subunit (**Figure 2g**). To provide an explanation for these unexpected findings, we examined the IL-17 dependence of G-CSF, a common target of the IL-17 pathway regulating neutrophil mobilization from the bone marrow. We found that G-CSF mRNA expression was strongly induced in response to OPC (data not shown) and high levels of G-CSF protein were present in the serum of infected mice (**Figure 2h**). However, G-CSF induction in response to *C. albicans* was independent of IL-17 (**Figure 2h**). Similarly, induction of the neutrophil chemotactic

factor CXCL1 was unchanged in mice lacking functional IL-17 signaling (**Supplementary Figure S2C**). In conclusion, these data clearly show that IL-17 is not essential for neutrophil mobilization from the bone marrow and trafficking to the infected tissue during OPC.

Neutrophil function is normal in IL-17-deficient mice

In the aim of identifying the underlying mechanism of IL-17-dependent antifungal defense in OPC, we tested whether IL-17 may impact the activity of neutrophils. As we have shown previously, IL-17 pathway deficiency is not associated with an intrinsic defect in candidacidal activity of neutrophils.⁴⁰ We therefore explored the role of granulocyte macrophage colony-stimulating factor, which has a key role in promoting the candidacidal activity of neutrophils during systemic candidiasis and thereby prevents mice from rapidly succumbing to intravenous infection with *C. albicans*.⁴⁰ In agreement with *Csf2*, the gene encoding granulocyte macrophage colony-stimulating factor, being a known target of the IL-17

pathway,^{21,25,26,41} its expression was induced during OPC in WT but not IL-17RC-deficient animals (**Figure 3a**). However, granulocyte macrophage colony-stimulating factor-deficiency did not cause impaired fungal control during OPC (**Figure 3b,c**) highlighting important differences in the mechanism of antifungal control during different forms of candidiasis. When directly evaluating the activation status of neutrophils in *Il17ra*^{-/-} mice and controls, we did not observe differences in the expression of the activation markers CD11b and CD18 (**Figure 3d**). Furthermore, the release of reactive oxygen species by neutrophils isolated from infected tongues was not affected by the lack of a functional IL-17 pathway (**Figure 3e,f**), despite the fact that mice deficient in gp91phox (encoded by *Cybb*), an essential subunit of the NADPH oxidase required for the generation of reactive oxygen species, were unable to clear the fungus (**Figure 3g**). Finally, we observed dispersed 4',6-diamidino-2-phenylindole dihydrochloride and extranuclear histone H1 staining in neutrophil-rich areas in infected tongues suggesting the presence of neutrophil extracellular traps, but we

Figure 3 Neutrophil activity is normal in absence of a functional interleukin-17 (IL-17) pathway. **(a)** *Csf2* mRNA induction in the tongue of wild type (WT) and *Il17rc*^{-/-} mice on day 1 post infection. Each symbol represents one mouse, the mean + s.d. is indicated. Data are pooled from two independent experiments. **P* < 0.05. **(b)** Fungal burden in the tongue of WT and *Csf2*^{-/-} mice on day 7 post infection. Each symbol represents one mouse; the dotted line represents the detection limit. **(c)** Body weight of WT and *Csf2*^{-/-} mice in response to infection. Data are the mean + s.d. of six animals and are pooled from two independent experiments. **(d)** CD11b and CD18 surface expression on CD11b⁺ Ly6G⁺ Ly6C⁺ neutrophils in the tongue and in the peripheral blood of *Il17ra*^{-/-} and heterozygous control mice on day 1 post infection. The same results were also observed in wild-type controls (not shown). Each symbol represents one mouse, the mean + s.d. is indicated. **(e, f)** Blood and tongue neutrophils from infected *Il17ra*^{-/-} and heterozygous control mice were incubated with dichlorofluorescein diacetate (DCF-DA) and dichlorofluorescein (DCF) levels were analyzed by flow cytometry. Representative stainings of CD11b⁺ Ly6G⁺ Ly6C⁺ neutrophils from infected mice are shown in **e**. Data from infected *Cybb*^{-/-} neutrophils are included as a negative control. Quantification of DCF^{hi} neutrophils is shown in **f**. Each symbol represents one mouse, the mean + s.d. is indicated. Data are representative of two independent experiments. **(g)** Fungal burden in the tongue of *Cybb*^{-/-} and wild-type control mice was analyzed on day 2 and day 3 post infection as indicated. Each symbol represents one mouse; the mean + s.d. is indicated. Data are pooled from two independent experiments.

Figure 4 Interleukin-17 (IL-17) deficiency allows persistent fungal colonization without overt inflammation. (a) Fungal burden in the tongue of wild type (WT), *Il-17ra*^{-/-}, and *Il-17rc*^{-/-} mice on day 7 post infection. (b) Body weight of WT, *Il-17ra*^{-/-}, and *Il-17rc*^{-/-} mice in response to infection. (c) Fungal burden in the tongue of WT and *Il17rc*^{-/-} mice on day 14 post infection. (d) Periodic-acid Schiff (PAS)/hematoxylin staining of sagittal tongue sections from WT and *Il17rc*^{-/-} mice on day 7 post infection. Representative sections are shown. Bar = 100 μ m. (e) CD11b⁺ Ly6G⁺ Ly6C⁺ neutrophils were counted in naïve and infected tongues of WT and *Il17rc*^{-/-} mice at the indicated time points. (f, g) Body weight (f) and fungal burden in the tongue (g) of infected WT mice on day 7 post infection that were treated with anti-IL-17A and anti-IL-17F starting from day -1 or from day +2 post infection onwards. The dotted line in a, c, and g represents the detection limit. In a, c, e, and g, each symbol represents one mouse, and the mean $\pm$ s.d. is indicated. In f, data are the mean $\pm$ s.d. of 9–10 animals. Differences between the treated groups and the untreated group are statistically significant from day 4 post infection onwards with $*P < 0.05$. Differences between the two treated groups are not statistically significant. Data shown in a, b, c, e, f, and g are pooled from at least two independent experiments.

were unable to detect differences between IL-17A- and IL-17F-depleted and non-depleted mice (data not shown). From these data, we concluded that although neutrophils appeared to be strongly activated during acute OPC, their contribution to protection seemed to be completely independent of the IL-17 pathway.

IL-17A and IL-17F are essential for fungal clearance in a neutrophil-independent manner

While IL-17 signaling defects do not impact the neutrophil response to OPC, as suggested by our results, the IL-17 pathway is strictly required for host protection and fungal clearance in the oral mucosa. In agreement with published reports,^{9,19,42} *Il17ra*^{-/-} and *Il17rc*^{-/-} mice failed to clear the fungus (Figure 4a). Instead they stabilized their body weight at a reduced level and adapted a constant colonization with *C. albicans* (Figure 4b,c and data not shown). *C. albicans* hyphae persisted in localized foci in the stratum corneum (Figure 4d). In response to the prolonged presence of the fungus, the epithelial architecture appeared strongly disrupted in vicinity of fungal foci, but the local response was no longer characterized by large inflammatory infiltrates after 1 week of infection. Neutrophil numbers were maximal on day 1 post infection and continuously decreased thereafter in both *Il17rc*^{-/-} and wild-type mice (Figure 4e). The slightly higher neutrophil counts in *Il17rc*^{-/-} mice observed on day 3 post infection and onwards

was most likely a consequence of the increased fungal burden in these mice at these later time points (see Figure 4a,c and data not shown). Next, we explored the time window, in which IL-17A and IL-17F were required for fungal control. For this, we administered neutralizing antibodies to wild-type mice to deplete IL-17A and IL-17F starting from day 2 post infection, a time point when the massive neutrophil response was already declining (see Figures 1a and 4e), whereas IL-17 production was still dependent on innate cellular sources.¹⁹ This prevented fungal clearance and kept the mice from regaining their initial weight (Figure 4f,g). In fact, the fungal load was only mildly reduced in these mice on day 7 post infection compared with mice depleted of IL-17A and IL-17F during the entire course of infection starting from day -1 (Figure 4g) suggesting that the IL-17 pathway remains critical for host protection beyond the immediate neutrophil-mediated response on day 1 post infection. Together, these data supported and expanded our previous conclusions and indicated that neutrophils were not sufficient for resolution of infection and that the clearance of *C. albicans* from the tissue was dependent on IL-17 and mediated by cells other than neutrophils. Experiments with bone marrow chimeric mice confirmed a role of radioresistant cells, most likely epithelial cells (data not shown).

To further explore the role of IL-17A and IL-17F in host protection from OPC, we turned to IL-17 target genes that we had not considered so far, namely AMPs. AMPs including

Figure 5 S100A9 expression in the epithelium but not in neutrophils is selectively regulated by the interleukin-17 (IL-17) pathway. (a) Sagittal tongue sections from infected wild-type (WT) mice were stained 1 day post infection with anti-S100A9 (green) and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (blue). Representative images are shown. Bar = 53 μ m. (b) S100A9 protein levels on day 1 post infection in tongue homogenates of WT (white bars) and *Il17rc*^{-/-} mice (gray bars). (c) S100A9 protein levels on day 1 post infection in tongue homogenates of WT (white bars) and *Il17ra*^{-/-} mice (gray bars) that were treated with anti-Ly6G and anti-G-CSF or left untreated as indicated. (d) S100A9 mRNA on day 1 post infection in the tongue of WT and *Il17ra*^{-/-} mice, which were treated as in c. Each symbol represents one mouse; the mean $\pm$ s.d. is indicated. Data shown in b and c are representative of two independent experiments, while data shown in d are pooled from two independent experiments. (e, f) Live CD45⁺ EpCAM⁺ CD31⁻ tongue epithelial cells (e) and CD45⁺ Ly6G⁺ neutrophils (f) were sorted from WT and *Il17ra*^{-/-} mice on day 1 post infection and S100A9 mRNA levels were determined. Data are the mean $\pm$ s.d. of replicate measurements from a pool of five animals per group. n.d., not detected. See also **Supplementary Figure S3**.

S100A8, S100A9, and defensin beta 3 are strongly induced during OPC.⁹ However, the molecular and cellular regulation of AMPs is not well understood. Here we focused on S100A9, a subunit of the protein complex calprotectin, which chelates divalent metal ions required for microbial growth⁴³ and which contributes critically to protection from *C. albicans* in several infection models.⁴⁴ We found that S100A9 protein accumulated in the oral mucosa in vicinity of invading *C. albicans* (Figure 5a). While S100A9 mRNA induction in total tongue preparations was strongly reduced in absence of a functional IL-17 pathway,⁹ we found that S100A9 protein levels were only partially reduced in total tongue homogenates of IL-17A- and IL-17F-depleted animals as well as IL-17RA- and IL-17RC-deficient animals compared with respective controls (Figure 5b and data not shown). We hypothesized that this discrepancy between mRNA and protein measurements may be explained by different modes of regulation in different cellular compartments. S100A9 is produced abundantly by neutrophils, which store preformed S100A9 protein in their

cytoplasm and release it during neutrophil extracellular trap formation to decorate expelled chromatin fibers.⁴⁴ In addition, S100A9 can also be expressed in non-hematopoietic cells such as epithelial cells under inflammatory conditions.⁴⁵ We hypothesized that S100A9 in the tongue of OPC-infected mice may be derived from at least two different cellular sources and that epithelial cell-derived but not neutrophil-derived S100A9 expression maybe under the control of the IL-17 pathway. Such a scenario would be consistent with our observation that total tongue S100A9 protein expression was only partially dependent on IL-17, whereas *de novo* S100A9 mRNA induction in response to *C. albicans* relied on a functional IL-17 pathway. To test this hypothesis, we depleted *Il17ra*^{-/-} mice of neutrophils before infection. This resulted in a complete loss of S100A9 protein compared with the partial reduction in neutrophil-sufficient *Il17ra*^{-/-} mice on day 1 post infection. In contrast, *de novo* synthesis of S100A9 mRNA was impaired in absence of IL-17 signaling independent of the presence or absence of neutrophils (Figure 5c,d). The same

was also observed for other AMPs such as defensin beta 3 (**Supplementary Figure S3**). The selective IL-17 dependence of AMP induction in epithelial cells, but not neutrophils, was further confirmed by cell-sorting experiments. While S100A9 mRNA was not detected in epithelial cells from *Il17ra* $-/-$ mice on day 1 post infection (**Figure 5e**), S100A9 transcripts were present in neutrophils from both wild type and *Il17ra* $-/-$ mice at comparable levels (**Figure 5f**). Together, these data revealed a cell type-specific regulation of AMPs including S100A9 and defensin beta 3 in neutrophils and non-hematopoietic cells. Epithelial cells, but not neutrophils, act as effector cells of IL-17-mediated protection from OPC. This is consistent with and strongly supports our findings that IL-17 acts in a neutrophil-independent manner to mediate antifungal immunity in the oral mucosa.

DISCUSSION

The biology of IL-17 called much attention since the first report on T helper-17 cells almost a decade ago,^{41,46} and even more since the discovery of IL-17-secreting ILCs.^{47,48} Given its potential to promote pathological conditions, IL-17 was investigated most extensively in the context of autoimmune and autoinflammatory diseases.¹² However, IL-17 has a crucial role in host protection from certain types of infections, first and foremost those caused by *C. albicans*, as revealed by the identification of genetic defects in IL-17-related genes in CMC patients.¹ While the focus of research has been concentrated on identifying the cellular sources of IL-17 in different settings, less effort was put on defining the mechanism by which IL-17 exerts its biological effects. Because common IL-17 target genes regulate neutrophil mobilization from the bone marrow and recruitment to the site of infection, the activity of IL-17 is generally considered to be linked to neutrophils. In the context of OPC, however, as we show here, the functions of IL-17 and neutrophils are distinct and they are uncoupled from one another. While neutrophils confine the fungus to the oral epithelium and, like a rampart, prevent fungal dissemination into the underlying tissue, IL-17 is essential for eliminating *C. albicans* from the host and for preventing fungal persistence in the epithelium in a neutrophil-independent manner. This study provides important new insights into IL-17-mediated antifungal defense mechanism acting in mucosal barrier tissues.

Most evidence for IL-17 dependency of neutrophil recruitment was obtained from pulmonary infection models.⁴⁹ In the skin, however, and in line with what we observed during OPC, IL-17-mediated effects appeared to be independent of neutrophils.⁵⁰ Further studies will be necessary to provide clarity about tissue-specific regulation of divergent IL-17-mediated activities. Our data are based on the analysis of five different settings with a defective IL-17 pathway, including *Il17ra* $-/-$, *Il17rc* $-/-$, IL-17A-, and IL-17F-depleted WT mice, *RORc* $-/-$ and ILC-depleted *Rag1* $-/-$ mice, which all provided comparable results. In contrast to published reports, which described reduced neutrophils in IL-17 receptor-deficient mice on day 5 post OPC infection,^{9,37,42} we did not find any changes in neutrophil numbers or distribution

when counting the cells in the entire tongue by flow cytometry and evaluating neutrophil distribution on histological sections. We focused our analysis on an early time point, when the impact of neutrophils on the control of infection was most relevant and when the fungal load was comparable between IL-17 pathway-deficient and control animals. From day 3 post infection onwards, we observed even higher neutrophil numbers in IL-17 receptor-deficient mice compared with controls, which was most likely the result of the persistent infection in absence of a functional IL-17 pathway. The detailed mechanism that instructs G-CSF-dependent neutrophil mobilization and recruitment during OPC awaits further investigations. Direct fungal recognition by the epithelium induces G-CSF production⁵¹ and may thus be sufficient to drive the neutrophil response during OPC.

The IL-17 family member IL-17C activates similar target genes as IL-17A and IL-17F,^{38,39} and by promoting psoriaform skin inflammation in mouse and Man, it displays overlapping effects with IL-17A and IL-17F.⁵² The strong induction of IL-17C during OPC suggested that this new IL-17 family member may also be involved in antifungal defense. However, our data indicate that this is not the case and that IL-17RE-dependent signaling does not contribute to protection from OPC. IL-17E, which we also investigated, was not induced during OPC, highlighting the importance of IL-17A and IL-17F as the main IL-17 family members in antifungal defense.

Neutropenia—in contrast to IL-17 deficiency—is generally not a specific risk factor for mucocutaneous candidiasis in humans. The situation in mice, where *C. albicans* is not part of the normal microflora, is different and neutrophils are strictly required to restrain the large fungal load that the host is exposed to under experimental conditions. Accordingly, the severity of disease is not comparable between neutrophil-depleted mice and IL-17-deficient mice. While *C. albicans* hyphae penetrated deep tissue in neutrophil-depleted conditions, the fungus was restricted to the stratum corneum and formed isolated foci during persistent colonization in IL-17 signaling-deficient animals. A similar distribution was observed in pseudomembranous candidiasis in human immunodeficiency virus-infected individuals,⁵³ which display low T-cell counts and in particular Th17 counts.⁴⁰ Despite the superficial location of *C. albicans* in the epithelium, the host does not succeed to clear the fungus in the absence of a functional IL-17 pathway. The need for IL-17 is continuous and is critical from the very beginning, as illustrated by the dependence on IL-17 production from innate cellular sources.¹⁹ Delayed depletion of IL-17A and IL-17F from day 2 post infection onwards resulted in only a limited reduction in fungal load compared with mice depleted throughout the entire period of infection and it prevented full recovery. Persistent colonization of the oral epithelium with *C. albicans* is not associated with enhanced inflammation or neutrophilia. This is in sharp contrast to the situation observed in systemic candidiasis, where uncontrolled fungus in infected kidneys elicits increased recruitment of neutrophils,^{54,55} and neutrophil accumulation (late after infection) is associated with immunopathology and mortality.^{56,57}

IL-17A has been proposed to bind directly to *C. albicans*.⁵⁸ However, such an interaction, which could potentially have negative consequences for the fungus, is unlikely to be involved in antifungal immunity at the oral mucosa, as the defect in controlling the fungus observed in mice lacking IL-17A (and IL-17F) cytokines is comparable with that in *Il17ra* $-/-$ and *Il17rc* $-/-$ mice expressing normal or even higher levels of IL-17. IL-17-dependent induction of AMPs during OPC has been reported previously,⁹ but their cellular source remained unclear as only bulk tongue tissue was analyzed. AMPs such as calprotectin and defensins are produced by different cell types including neutrophils and epithelial cells. Here, we dissected the AMP response and demonstrated that IL-17-driven AMP expression is uncoupled from the neutrophil response. S100A9 production by epithelial cells, which is regulated at the level of transcription, depends selectively on IL-17 signaling in the oral mucosa. In contrast, S100A9 secretion by neutrophils, which is regulated posttranslationally, is independent of IL-17. This result highlights the critical role of the oral epithelium in protective immunity against OPC. The contribution of individual AMPs to fungal clearance remains difficult to analyze conclusively due to a lack of suitable tools. Furthermore, AMPs display a high degree of redundancy, which renders the innate immune system very robust. Accordingly, selective deficiency in Lipocalin 2 does not abolish the resistance of mice to OPC.⁵⁹ The critical role of calprotectin in antifungal defense on the other hand has been demonstrated by the reduced fungal clearance and enhanced mortality of S100A9-deficient mice in response to *C. albicans* infections including the skin.⁴⁴ In line with the murine studies, human immunodeficiency virus-positive individuals, which are prone to develop CMC and in particular OPC, show reduced levels of calprotectin in saliva.⁶⁰ Together, this strongly suggests that the critical role of the IL-17 pathway for protective immunity against *C. albicans* is to instruct the antifungal response of the epithelium and that the IL-17-driven activity is fully uncoupled from the neutrophil response in the oral mucosa.

METHODS

Mice and depletion strategies. C57Bl/6 mice were purchased from Janvier Elevage (Saint Berthevin, France). *Il17ra* $-/-$, *il17rc* $-/-$, and *il17re* $-/-$ mice were obtained from Amgen (Thousand Oaks, CA) and bred at our animal facility Rodent Center HCI. *Rorc* $-/-$, *Csf2* $-/-$, and *Cybb* $-/-$ mice were a gift from Burkhard Becher (University of Zürich, Switzerland), Manfred Kopf and Wolf-Dietrich Hardt (both ETH Zürich, Switzerland), respectively. All mice were on the C57Bl/6 background, were kept in specific pathogen-free conditions, and were used at 6–15 weeks of age. In some experiments, anti-IL-17A (clone 17F3, BioXCell (West Lebanon, NH), 200 μ g per mouse per day) and anti-IL-17F⁶¹ (kindly provided by Pfizer (New York, NY), 200 μ g per mouse per day) was administered daily starting from 1 day before infection. For neutrophil depletion, NimpR14 (produced from the hybridoma, which was kindly provided by Nancy Hogg, Cancer Research UK, London) was administered daily starting from one day before infection. Alternatively, anti-GSF (clone 67604, R&D Systems (Abingdon, UK), 10 μ g per mouse per day) was administered daily starting from one day before infection together with one dose of anti-Ly6G (clone 1A8, BioXCell, 150 μ g per mouse) on day -1 . All antibodies were administered via the intraperitoneal

route. All mouse experiments described in this study were conducted in strict accordance with the guidelines of the Swiss Animal Protection Law and were performed under protocols approved by the Veterinary office of the Canton Zürich, Switzerland (license number 184/2009 and 201/2012). All efforts were made to minimize suffering and ensure the highest ethical and humane standards.

Fungal strain and infection. The *C. albicans* laboratory strain SC5314 was grown in yeast peptone dextrose medium at 30 °C for 15–18 h. Mice were infected with 2.5×10^6 c.f.u. *C. albicans* sublingually as described⁶² without immunosuppression. Mice were monitored for morbidity and killed when they showed severe signs of pain or distress. The loss and recovery of body weight were monitored throughout the course of all experiments and is displayed as % of initial weight. For determination of fungal burden, the tongue was removed, homogenized in sterile 0.05% NP40 in H₂O for 3 min at 25 Hz using a Tissue Lyzer (Qiagen, Hombrechtikon, Switzerland), and serial dilutions were plated on yeast peptone dextrose agar containing 100 μ g ml⁻¹ ampicillin.

Isolation of tongue cells and analysis by flow cytometry. Mice were anesthetized with a sublethal dose of ketamin, xylazin, and acepromazin, and perfused by injection of phosphate-buffered saline into the right heart ventricle. Tongues were cut into fine pieces and digested with DNase I (2.4 mg ml⁻¹, Roche, Rotkreuz, Switzerland) and collagenase IV (4.8 mg ml⁻¹, Life Technologies, Zug, Switzerland) in phosphate-buffered saline for 45 min at 37 °C. Single-cell suspensions were stained in ice-cold phosphate-buffered saline supplemented with 2 mM EDTA, 10% fetal calf serum, and 0.02% sodium azide with CD45 (clone 104, Biolegend, San Diego, CA), CD11b (clone M1/70, Biolegend), Ly6C (clone AL-21, BD Biosciences, Allschwil, Switzerland), Ly6G (clone 1A8, BD Biosciences), CD18 (M18/2, Biolegend), EpCAM (G8.8, Biolegend), and CD31 (clone MEC13.3, Biolegend). Data were acquired on a LSRII (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, OR). An Aria III (BD Biosciences) was used for cell sorting.

RNA isolation and quantitative reverse transcriptase-PCR. Isolation of total RNA from tongues and generation of cDNA was carried out according to standard protocols. Quantitative PCR was performed using SYBR Green (Roche) and a Rotor-Gene 3000 (Corbett Research/Qiagen). The primers were *Cxcl1* fwd 5'-ccgctcgtctctgtg-3', *Cxcl1* rev 5'-gcagctcattggcgatag-3'; *S100a9* fwd 5'-gtccaggtcctccatgatg-3' and *S100a9* rev 5'-tcagacaatggtggaagca-3'; *defb3* fwd 5'-gtctccactgca gcttttag-3' and *defb3* rev 5'-actgcaatctgacagtggtt-3'; *Il17c* fwd 5'-ctggaagctgacactcacg-3' and *Il17c* rev 5'-ggtagcggttctatctgtg-3'. Data were normalized to β -actin and displayed as relative values.

Tissue homogenization and enzyme-linked immunosorbent assay. For protein measurements, tongues were homogenized in phosphate-buffered saline with 0.1% Triton-X100 and Roche Protein Inhibitor tablet using a Tissue Lyzer (Qiagen) for 3 min at 25 Hz. G-CSF protein was determined by sandwich enzyme-linked immunosorbent assay using purified anti-G-CSF (clone 67604, R&D Systems) for coating and biotinylated polyclonal rabbit anti-G-CSF (Peprotech, London, UK) for detection according to standard protocols. S100A9 protein levels were determined with the S100A9 DuoSet enzyme-linked immunosorbent assay kit (R&D Systems).

Histochemistry and immunofluorescence. Tissues were embedded in OCT (Sakura, Alphen aan den Rijn, The Netherlands), snap-frozen in liquid nitrogen, and stored at -20 °C. Sagittal cryosections (10 μ m) were air dried at room temperature, stained with periodic-acid Schiff (Roth, Arlesheim, Switzerland), and counterstained with haematoxylin (Roth). Sections were mounted with Pertex (Biosystems, Nunningen, Switzerland) and analyzed on an Axiovert 25 (Zeiss, Feldbach, Switzerland) at $\times 10$ – 40 magnification. All scale bars indicate 100 μ m. For immunofluorescence stainings, cryosections

were fixed with ice-cold 4% paraformaldehyde and stained with anti-S100A9 (kindly provided by C. Urban, Umea, Sweden, $0.5 \mu\text{g ml}^{-1}$), anti-mouse-IgG-Alexa647 ($5 \mu\text{g ml}^{-1}$, Abcam, Cambridge, UK), anti-rat-IgG(H + L)-Alexa488 ($7.5 \mu\text{g ml}^{-1}$, Jackson ImmunoResearch, Newmarket, UK), and 4',6-diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich, Buchs, Switzerland). Slides were mounted with Mowiol (VWR International AG, Dietikon, Switzerland). Images were acquired with a Zeiss Axiovert 200-m microscope, a CSU-X1 spinning-disc confocal unit (Visitron Systems, Puchheim, Germany) and an Evolve 512 EMCCD camera (Photometrics, Tucson, AZ). Objectives of $\times 10$ and $\times 20$ were used. Image analysis was performed with Volocity software (Perkin Elmer, Schwerzenbach, Switzerland).

Oxidative burst assay. Neutrophils were incubated with $20 \mu\text{M}$ dichlorofluorescein diacetate (Sigma-Aldrich) in Ca^{2+} - and Mg^{2+} -free medium at 37°C for 15 min, washed with RPMI, and stimulated with 5×10^4 heat-killed *Candida* before the cells were stained with cell surface antibodies and analyzed by flow cytometry.

Statistics. Statistical significance was determined by non-parametric Mann–Whitney *U*-test using GraphPad Prism (GraphPad Software, La Jolla, CA) with $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. For data plotted on a logarithmic scale, the geometric mean is indicated.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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AUTHOR CONTRIBUTIONS

K.T.-W., A.G., and S.L.-L. conceived and designed the experiments. K.T.-W., A.G., S.N., and P.D. performed the experiments. K.T.-W., A.G., S.N., P.D., and S.L.-L. analyzed the data. K.T.-W., A.G., and S.L.-L. wrote the paper.

DISCLOSURE

The authors declared no conflict of interest.

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