Mechanistic Insights into the Roles of IL-22/IL-22RA1 Axis in Oral Antifungal Immunity

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Oropharyngeal candidiasis (OPC, oral thrush) is an opportunistic infection caused by the commensal fungus *Candida albicans*. IL-17 and IL-22 are produced by Type 17 lymphocytes. Both cytokines mediate antifungal immunity yet activate quite distinct downstream signaling pathways. While much is now understood about how IL-17 promotes immunity in OPC, the activities of IL-22 are far less well delineated. In this dissertation, I focused on identifying mechanisms by which IL-22 mediates oral antifungal immunity. In chapters 3 and 4, I identified key differences between IL-22 and IL-17-dependent antifungal events during innate OPC response. We show that, despite having similar requirements for induction from Type 17 cells, IL-22 and IL-17 function non-redundantly during OPC. We find that the IL-22 and IL-17 receptors are required in anatomically distinct locations within the oral mucosa; loss of IL-22RA1 or STAT3 in the oral basal epithelial layer (BEL) causes susceptibility to OPC, whereas IL-17RA is needed in the suprabasal epithelial layer (SEL). Transcriptional profiling of the tongue linked IL-22/STAT3 to oral epithelial cell proliferation and survival, but also, unexpectedly, to driving an IL-17-specific gene signature. We show that IL-22 mediates regenerative signals on the BEL that replenish the IL-17RA-expressing SEL, thereby restoring the ability of the oral epithelium to respond to IL-17 and thus to mediate antifungal events. Consequently, IL-22 signaling in BEL 'licenses' IL-17 signaling in the oral mucosa, revealing spatially distinct yet cooperative activities of IL-22 and IL-17 in oral candidiasis. In chapter 5, I assessed the roles of IL-22 in T cell recall OPC settings. IL-22 is protective in the adaptive response against OPC although the contribution

of innate IL-22-mediated responses could not be ruled out. While IL-22 deficiency resulted in cervical lymph node (cLN) hypertrophy, there was no evidence of *C. albicans* translocation to the cLN or increased proliferative expansion of Th17 cells. Loss of IL-22 resulted in increased Th17 ($CD4^+IL\text{-}17A^+$) but decreased Th1($CD4^+IFN\text{-}\gamma^+$) frequency. Hence IL-22 may be acting on the cLN stroma to modulate T cell responses in recall OPC settings. Our findings have implications for antifungal vaccine design strategies and oral immunosurveillance mechanisms against oral pathologies and extra-oral diseases.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AIDS – Acquired Immune Deficiency Syndrome

AhR – Aryl Hydrocarbon Receptor

AIRE – Autoimmune Regulator

AMP – Antimicrobial Peptides

APS-1 – Autoimmune Polyglandular Syndrome Type 1

APECED – Autoimmune Polyendocrinopathy Candidiasis Ectodermal Dystrophy

APC – Antigen Presenting Cells

ASC – Apoptosis Associated Speck-Like Protein

BEL – Basal Epithelial Layer

CFU – Colony Forming Unit

cLN – cervical Lymph Nodes

CLR – C-type Lectin Receptors

CMC – Chronic Mucocutaneous Candidiasis

DAMPs- Danger Associated Molecular Patterns

DC – Dendritic Cells

EGFR – Epidermal Growth Factor Receptor

FBS – Fetal Bovine Serum

GSEA- Gene Set Enrichment Analysis

H&E – Hematoxylin & Eosin

HIES – Hyper IgE Syndrome

HK – Heat-Killed

IF – Immunofluorescence

IFNλ –Interferon gamma

I.P. – Intraperitoneally

I.V. – Intravenous

IACUC – Institutional Animal Care and Use Committee

ICS – Intracellular Cytokine Staining

IL-17RA – Interleukin-17 Receptor A

IL-17RC – Interleukin-17 Receptor C

IL-22RA1 – Interleukin-22 Receptor A1

IL-22RA2 – Interleukin-22 Receptor A2

IL-1R – Interleukin-1 Receptor

IPA- Ingenuity Pathway Analysis

iNKT – invariant Natural Killer T Cells

MR – Mannose Receptor

NBF – Normal Buffered Formalin

NLR – Nod-Like Receptors

OECs – Oral Epithelial Cells

OPC – Oropharyngeal Candidiasis

PAMP – Pathogen Associated Molecular Patterns

PAS – Periodic Acid Schiff

PBMC – Peripheral Blood Mononuclear Cells

PBS – Phosphate Buffered Saline

PMA – Phorbol Myristate Acetate

PRR – Pattern Recognition Receptors

Rag 1 – Recombination Activating Gene 1

SEL – Suprabasal Epithelial Layer

STAT – Signal Transducers and Activators of Transcription

TCR – T Cell Receptor

Th – T helper

TLR – Toll-Like Receptor

TNF- Tumor Necrosis Factor

Tregs – Regulatory T Cells

VVC – Vulvovaginal Candidiasis

YPD – Yeast Peptone Dextrose

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1.0 Introduction

1.1 Candida and Candidiasis

Fungal infections are on the rise and remain a serious threat to public health. Yet, to date, there are no licensed antifungal vaccines. Among human pathogenic fungi, *Candida* species constitute the major cause of disease. Of the major *Candida* species, (namely, *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, *Candida krusei and Candida aureus*), *Candida albicans* is the most prevalent cause of infections [1, 2].

Candidiasis manifests mainly as mucocutaneous or invasive/disseminated disease. Mucocutaneous candidiasis includes superficial infections of the skin, nails, vaginal tract and oral cavity. Candidiasis of the oral cavity [also known as oral thrush or oropharyngeal candidiasis (OPC)] remains the most common human fungal infection [1-3]. *C. albicans* is a dimorphic fungus which exists as a commensal organism on healthy host mucosal surfaces such as the skin and mouth with about 50-80% carriage. However, perturbations in oral immunity as observed in immunosuppression associated with transplant recipients, radiotherapy and chemotherapy in head and neck cancer patients and steroid use in asthma patients can promote its incidence. Additionally, denture use, collateral damage from the use of biologics against protective cytokines, decreased CD4 count in HIV/AIDS and some congenital immunodeficiency syndromes are risk factors associated with oral thrush. During these perturbations, *C. albicans* transitions into its virulent hyphal state to cause disease [4, 5]. Mucocutaneous candidiasis can also develop as persistent or recurrent superficial infections of the skin, nails and mucosal membranes [6]. Vulvovaginal candidiasis (VVC) presents as burning or itchy sensations of the female genitalia often

accompanied by white vaginal discharge. As many as 75% of females may have at least one episode of VVC during their lifetime, while another 5% may experience recurrent or chronic episodes of vaginal irritation [7, 8]. Other patients experience invasive or disseminated candidiasis (generally referred to as candidemia) which may occur when *Candida* breaches the host epithelia and gains access to the blood stream. Candidemia may also occur after surgical procedures due to carriage on surgical tools. Candidemia is the second most common nosocomial infection and accounts for about 40% mortality [9]. These statistics reveal the enormous burden of *Candida* on human health.

1.2 Host *C. albicans* interactions and immunity

1.2.1 *Candida* virulence factors

The morphological plasticity of *C. albicans* to switch from yeast to hyphae is a key determinant of virulence and driver of the host immune response. In the presence of competent host immune responses, *C. albicans* yeast colonize mucosal surfaces without causing overt disease. In the absence of effective host immunosurveillance and increased fungal load, *C. albicans* produces hyphal filaments that invade host tissue to cause disease. Consequently, mutant strains of *C. albicans* that fail to form hyphal filaments are non-invasive and avirulent [10, 11]. Hyphae formation is therefore a key *C. albicans* virulence trait. Hyphae formation is characterized by the expression of hypha-associated virulence factors [such as hyphal wall protein 1(Hwp1p), secreted aspartyl protease (Sap) family proteins, agglutinin-like sequence 3 protein (Als3p), extent of cell

elongation 1 protein (Ece1p) and hyphal regulated 1 protein (Hyr1p)], which are important for hyphae adhesion, invasion and damage of host cells.

In the initial stages of infection, *C. albicans* expresses specific protein adhesins to promote attachment to epithelial surfaces. The best characterized *C. albicans* adhesins belong to the agglutinin-like sequence (Als) protein family which consist of 8 members (Als1p-Als7p and Als9p). While *C. albicans* strains lacking *Als1p*, *Als2p*, *Als3p* and *Als4p* failed to adhere to epithelial cells [12-14], *Als5*-, *Als6*- and *Als7*-deficient strains showed enhanced adhesion to epithelial cells [15]. The expression of these adhesins therefore appears to have opposing consequences and may either favor colonization or invasion of host cells. Hyphal wall protein 1 (Hwp1p) is important for *C. albicans* adherence as *Hwp1* deficient *Candida* failed to cause OPC in mice [16-18]. Once *Candida* is strongly attached, it begins to invade host epithelial cells.

Epithelial invasion is achieved through induced endocytosis or active penetration [19]. During endocytosis, *C. albicans* proteins Als3p and Ssa1p engage epithelial E-cadherin, epithelial growth factor receptor (EGFR) and human EGF receptor 2 (HER2) to activate the clathrin dependent endocytosis machinery [20-23]. Epithelial aryl hydrocarbon receptor (AhR) also mediates *C. albicans* endocytosis through EGFR activation [24]. Unlike fungal endocytosis which is driven by the host, active penetration is dictated by hyphae which must penetrate epithelial cells and or circumvent through intraepithelial junctions to breach the mucosal barrier [25, 26]. Although the exact mechanism of active penetration is not delineated, hyphal extension, physical force exerted by extending hyphae and the secretion of hydrolytic enzymes such as secreted aspartyl proteinases (Saps), phospholipases and lipases tend to facilitate the process [19, 27]. While Saps are known to promote degradation of host glycoproteins including mucins and E-cadherins [27, 28], their functional relevance in active hyphal penetration remains controversial,

since Sap1-6 failed to play critical roles in *C. albicans* invasion [29, 30]. Similarly, *C. albicans* phospholipase PLB1 degrades phospholipids present in host epithelial cell plasma membranes to facilitate penetration. However, the role of PLB1 remains controversial because PLB1 deficient *C. albicans* did not lose the ability to invade host epithelial cells [25, 31]. Like the phospholipases, *C. albicans* lipases encoded by LIP1-10 gene family are implicated in fungal invasion [32]. However, their functional relevance remains uncertain, as multiple groups have reported opposing effects of impaired lipase activity or deficiency on *C. albicans* invasion [25, 33].

In the context of OPC, successful *C. albicans* invasion is accompanied by destruction and sloughing of the superficial oral epithelium by secreted fungal factors that induce damage to the oral epithelium [27]. The *C. albicans* gene *ECE1* has emerged as a major damage-inducing factor during mucosal *Candida* infection [34]. While *ECE1*-deficient *C. albicans* are able to form hyphae, adhere to and invade epithelial cells, they fail to damage host epithelium [34]. *ECE1* encodes a 271 amino acid protein (Ece1p) that is cleaved by the endoproteinase Kex2p to generate eight distinct peptides [35]. The fragment spanning Ece1p62-93 is further processed by the carboxypeptidase Kex1p through terminal Arginine removal to generate the mature peptide, known as Candidalysin (Ece1p62-92). Candidalysin is a cytolytic peptide secreted by *C. albicans* hyphae during mucosal invasion and it is thought to signal through the epidermal growth factor receptor (EGFR) [36]. Similar to *ECE1* mutant strains, candidalysin-deficient *C. albicans* failed to cause epithelial damage and pro-inflammatory responses during mucosal invasion [34]. Both strains were also impaired in the ability to induce IL-17A from host lymphocytes [37]. Candidalysin has therefore emerged as a critical virulence factor important for *C. albicans* pathogenesis and immune activation at mucosal surfaces.

1.2.2 Recognition of *C. albicans*

Recognition of invading microbes is achieved through host pattern recognition receptors (PRRs) which interact with conserved microbe specific pathogen associated molecular patterns (PAMPS) or danger associated molecular patterns (DAMPs) released from infected or damaged cells and tissues [38]. The *C. albicans* cell wall serves as a rich source of PAMPs for PRRs. The masked inner skeleton is mainly composed of β-(1,3)-glucan and chitin moieties, which are anchored to cell wall proteins (mannoproteins) and mannans in the outer layer (**Fig.1.1**). Alterations in the expression and or localization of these cell wall components during yeast to hyphal transitions prompt the immune system to distinguish between the commensal yeast and pathogenic hyphal forms.

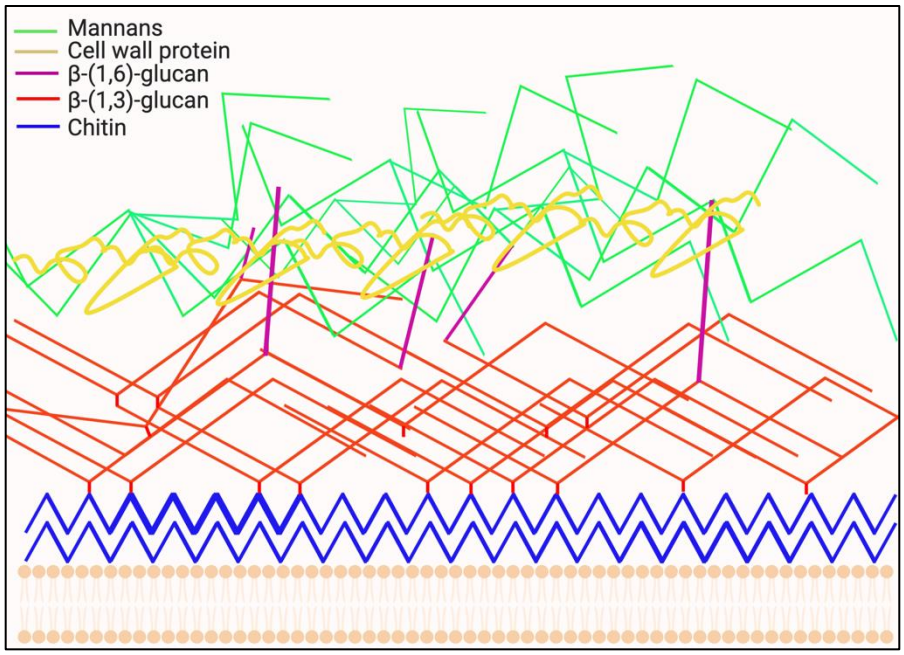

Figure 1.1: Structure of *C. albicans* cell wall

The major cell wall components of *C. albicans* cell wall are shown. Chitin and β-(1,3) glucan are buried deeper and closer to the outer cell membrane. Cell wall proteins connect β-(1,3) glucan residues to β-(1,6) glucan and mannan residues.

Invading hyphae lyse host epithelial cells, which then release DAMPs (IL-1α, IL-1β, S100A8/9) that promote the expansion of 'natural' Th17 (nTh17) cells [37]. In addition, direct engagement of PAMPs through PRRs trigger the secretion of cytokines including IL-1β, IL-23 and IL-6. These cytokines promote DC maturation and antigen presentation and polarize naïve CD4 T cells into Th17 cells (**Fig. 1.2**) [38-40]. Toll like receptors (TLR), C-type lectin receptors (CLR), and nucleotide binding domain leucine-rich receptors (NLRs) are the main families of PRRs implicated in antifungal immunity. While the respective *Candida*-derived PAMPs necessary for fungal recognition by TLRs and CLRs have been defined, PAMPs for NLRs have not been definitively identified (Summarized in **Table 1.1**).

Among the TLRs, TLR2 and TLR4 are the major PRRs in anti- *Candida* immunity. There are mixed reports on the role of TLR2 in antifungal immunity. While TLR2 ligation promotes protective proinflammatory responses in some settings [41-44], TLR2 tends to promote immunosuppression and *Candida* dissemination in other settings [45]. TLR4, on the other hand, is protective against disseminated candidiasis [46], and there is no clear requirement for TLR1, TLR6 and TLR9 in anti-*Candida* immunity [39, 47]. In line with the role of TLRs and IL-1R signaling in antifungal immunity, mice lacking Myd88 (a central adaptor downstream of TLR and IL-1R signaling) are susceptible to *Candida* infection. Like IL-1, IL-36, another member of the IL-1 family of cytokines requires Myd88 to mediate antifungal immunity [48]. However, mutations of MyD88 in humans are not associated with fungal infections and may instead suggest a more prominent role for other fungal recognition pathways.

The CLRs (dectin 1, dectin 2, mincle and mannose receptor) (**Table 1.1, Fig. 1.2**) and their downstream adaptors (CARD9/Bcl-10/MALT1) are implicated in anti-*Candida* immunity in both mice and humans [49-52]. Importantly, while *Card*$^{-/-}$ mice are susceptible to OPC in adaptive settings with impaired IL-17 induction, *Card*$^{-/-}$ mice are resistant to OPC in innate settings [53]. Hence other factors including candidalysin and IL-23 may be more critical than CLRs in fungal recognition during acute *C. albicans* exposure.

The intracellular inflammasome (mainly composed of NLRP1, NLRP3, NLRP10 and NLRC4, their adaptors, and proteases) also contributes to antifungal immunity by recognizing yet-to-be identified fungal PAMPS. Activation of the inflammasome promotes caspase-1-dependent cleavage and secretion of IL-1β and IL-18. While IL-1β promotes Th17 differentiation, IL-18 polarizes naïve CD4 T cells into Th1 cells [54, 55]. Consequently, *NLRP3*$^{-/-}$, *NLRP10*$^{-/-}$, *NLRC4*$^{-/-}$, *Il1r*$^{-/-}$ and *ASC*$^{-/-}$ are all sensitive *to Candida* infection and are associated with impaired IL-17 and IFN-γ production [41, 55-57].

Although most of these studies focused mainly on PAMP recognition through PRRs on APCs, emerging evidence supports a role of epithelial PRRs as well. β-glucans have been found to activate the epithelial receptor tyrosine kinase Ephrin A2 (EphA2) to induce proinflammatory antifungal responses [58]. Regardless of the cell types, efficient recognition of *Candida* and subsequent priming of protective Th17 responses may be dependent on the collective ability of PRRs to engage different fungal cell wall components to elicit appropriate stimuli to balance the onset and resolution of inflammation.

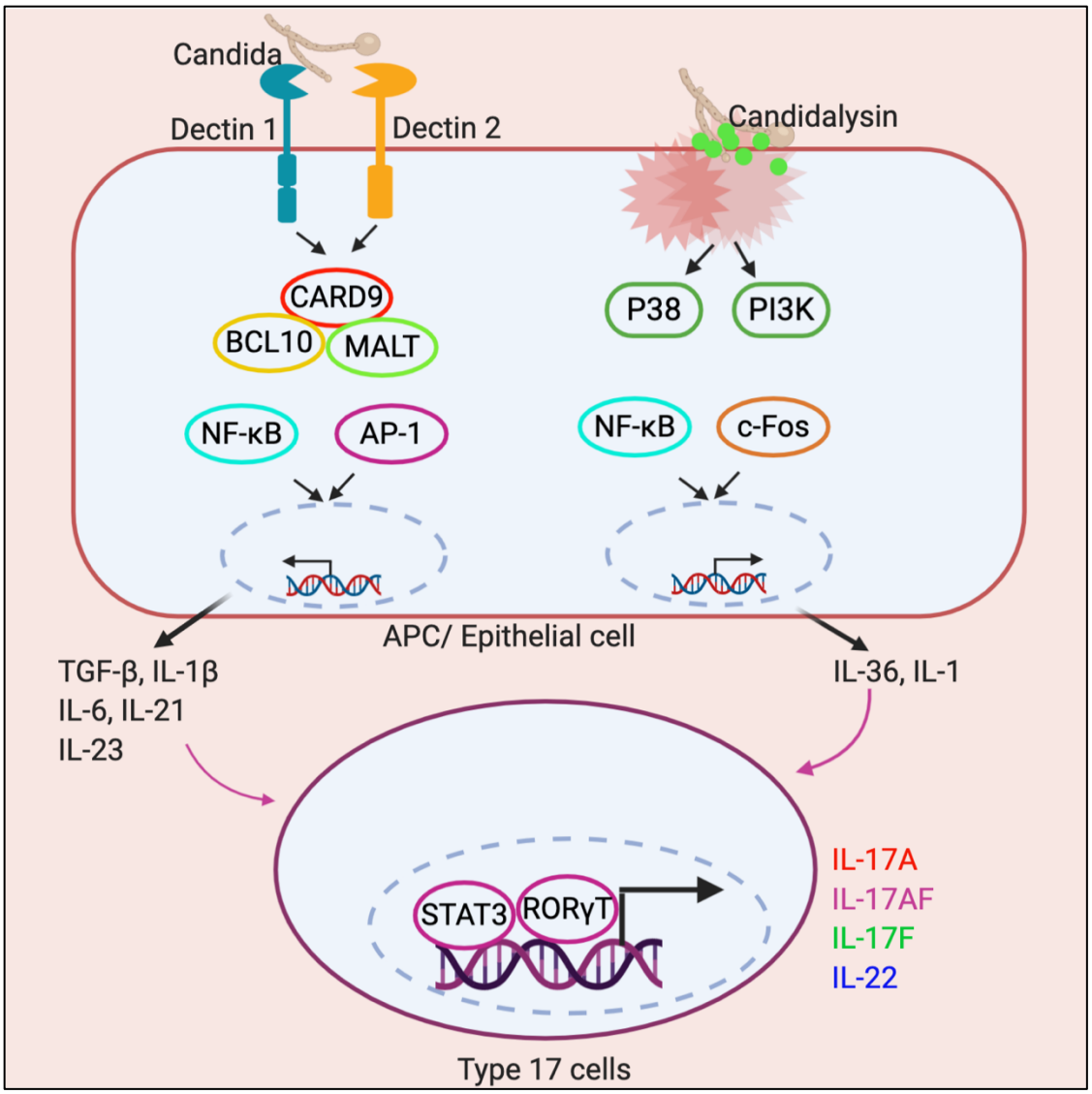

Figure 1.2: Activation of oral mucosal type 17 response to *C. albicans*

(A) Phagocytes recognize *C. albicans* via PRRs (Dectin1/2) leading to the production of Th17 polarizing cytokines. These cytokines activate STAT3 in type 17 cells to upregulate RORγT leading to the production of IL-17A, IL-17AF, IL-17F and IL-22. **(B)** Candidalysin secreted from *C. albicans* hyphae during oral epithelial cell invasion triggers the activation of c-Fos via p38-MAPK pathway along with NF-κB and PI3 kinase signaling pathway. These signals lead to the induction of IL-36 and IL-1 which target both epithelial cells and Type 17 cells to enhance Type 17 response.

Table 1.1: Summary of host PRRs and PAMPS in anti-fungal immunity

Family	PRR	Fungal PAMP	Reference
Toll like receptors (TLR)	TLR2	Phospholipomannan, β glucan	[42, 59, 60]
	TLR3	Double-stranded RNA	[61]
	TLR4	O-linked mannan	[46, 62]
	TLR9	DNA CPG motifs	[63]
C-type lectin receptors (CLR)	Dectin-1	β-1,3-glucan	[39, 64]
	Dectin-2	High mannose structures, α mannans	[65, 66]
	Mannose receptor	Mannan	[67]
	Mincle	High mannose structures	[68]
	Galectin-3	β-1,2-mannosides	[69]
	DC-SIGN	High mannose structures	[70]
Nod-like receptors (NLR)	NLRP3	DAMPS?	[56]
	NLRC4	DAMPS?	[57]
	NLRP10	DAMPS?	[55]
Others	EphA2	β-glucans	[58]

1.2.3 The oral epithelium; more than just a physical barrier

The oral cavity is the gateway to the GI tract and also partly connects the nasopharyngeal passage. Hence the oral cavity is a major connection to the head, digestive and respiratory tract. Unlike the lung and skin that can become fibrosed in response to pathological insults and persistent inflammation, the oral mucosa is adept at responding to insults (including invading pathogens, food and allergens, mechanical stress etc.) and restrains excessive inflammation [3, 71]. Furthermore, compared to the skin, the oral mucosa has intrinsic abilities to heal and repair faster. These features are partly attributed to its unique abilities to regulate inflammation, modulate stem cell, proliferation and differentiation programs while maintaining efficient remodeling of oral epithelial cells (OECs) [72, 73]. Moreover, the oral mucosa is poised at basal state and primed to respond to oral mucosal assaults [71]. The oral epithelium is therefore a formidable physical barrier with enormous capacity not only to limit microbial invasion and dissemination but also to instigate a myriad of immunosurveillance signaling cascades to limit invading pathogens and protect underlying tissues.

The organization and structure of the oral mucosa is key to its functions. The stratified squamous epithelia of the oral mucosa consists of specialized mucosa (tongue papillae, taste buds), lining mucosa (inner lip surface, buccal mucosa, soft palate and the floor of the mouth) and the masticatory mucosa (gingiva, hard palate, and dorsum of tongue) (**Fig. 1.3**) [3, 74]. These specialized mucosae have sensory abilities that are facilitated by motor and sensory nerve innervation to allow movement and perception of stimuli including taste, irritation and heat. The masticatory mucosae are distinguished from the lining mucosa by virtue of their toughness and resistance to stress. While the masticatory mucosae are keratinized to deal with persistent exposure

to physical stress, the lining mucosa are less subject to physical stress and are accordingly nonkeratinized or only partly keratinized [3, 75, 76].

Depending on the cell type and stage of differentiation, OECs express unique pairs of keratins which function as cytoskeletal proteins to maintain the integrity of the oral epithelium [75]. The oral epithelium is compartmentalized mainly into the underlying basal epithelial layer (BEL), an intermediate layer, and a post-mitotic overlying suprabasal epithelial layer (SEL) (**Fig. 1.3**).

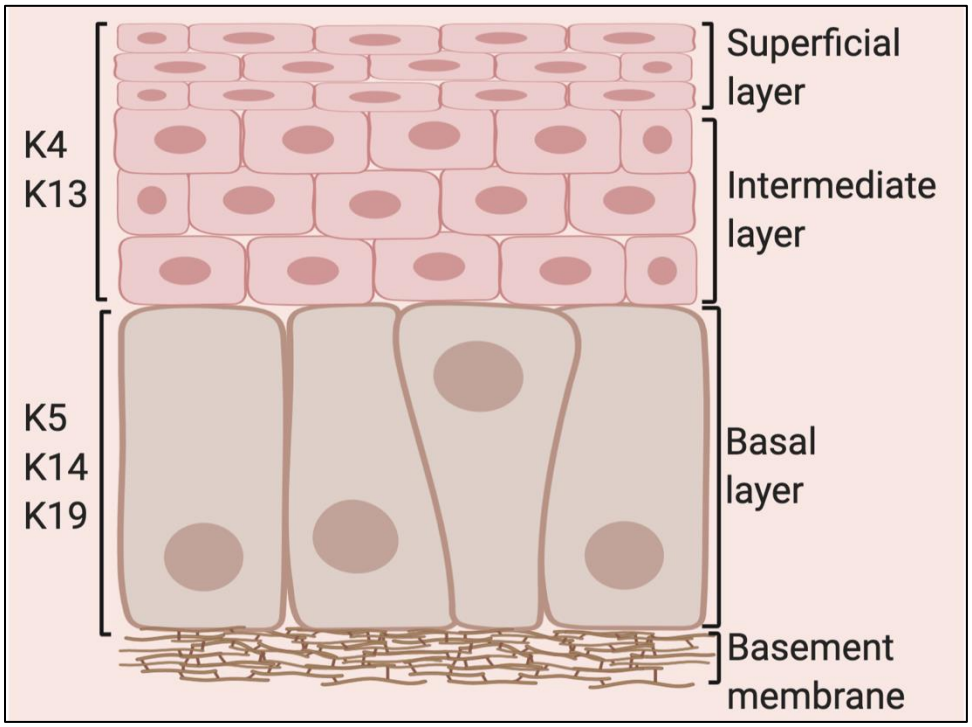

Figure 1.3: Histology of the oral mucosa

The oral cavity is subject to allergenic, masticatory and pathogenic stress and the stratified squamous epithelium of the oral cavity is adapted to respond to these insults. Keratinizing epithelium of the tongue is made of 2 main layers, which express unique sets of keratins; K4 and K13 in the supra basal epithelial layer and K5, K14 and K19 in the basal epithelial layer. The dorsum of the tongue also has several papillae containing taste buds for taste sensation.

The proliferative basal epithelial cells of most epithelial surfaces including the oral mucosa mostly express keratin 5 (K5) and keratin 14 (K14) and harbor epithelial stem cells. Emerging studies have shown the presence of $Lrig1^+$ and $Lrig5^+$ stem cells in the oral basal epithelium [77-79]. Cells of the BEL undergo a program of coordinated self-renewal and differentiation to give rise to terminally differentiated cells, which replenish the overlying SEL [80-82]. The oral SEL, on the other hand, mostly expresses keratin 4 (K4) and keratin 13 (K13). Additional keratins including keratin 6 (K6), keratin 16 (K16), and keratin 2p (K2P) that mark the differentiated epithelium are expressed in the SEL [80]. The differentiated SEL makes the first contact with invading hyphae and is damaged during invasion. The proliferation and subsequent differentiation of the basal epithelial cells is therefore required to replenish, maintain and restore oral mucosal integrity and immunity after infection or injury.

Oral epithelial cells are key to oral immunity and are important for recognizing and instigating early immune responses against microbes [83]. During OPC, oral epithelial cells sense the transition of *C. albicans* from commensal yeast to virulent hyphae to dictate downstream signaling cascades [34]. This early recognition is mediated through receptors including HER2/Neu and EGFR [23]. EGFR binds to candidalysin to trigger epithelial damage and cFOS mediated host immune responses [34, 36, 37]. This immunosurveillance response of OECs to *C. albicans* promotes the induction of innate cytokines (including IL-1α/β, IL-36, CCL20, G-CSF (granulocyte colony-stimulating factor), GM-CSF (granulocyte-macrophage colony-stimulating factor), and antimicrobial peptides (β-defensins, S100A8/9) [37, 84, 85]. This tissue microenvironment promotes IL-17 expression and proliferation of IL-17 producing natural Th17 ("nTh17") cells [37, 86]. IL-17 drives canonical NF-κB pathway and MAPK (mitogen activated protein kinase) cascades to further promote the induction of neutrophil-recruiting factors, myeloid and lymphoid

chemoattractants and antimicrobial peptides [87]. In particular, IL-17RA signaling in the SEL is critical for IL-17-mediated protection and induction of *DefB3* (β-defensin 3) during OPC [74, 88]. Thus, by virtue of its structure and strategic positioning, the oral epithelium is able to recognize and initiate a cascade of immunosurveillance programs to respond to oral *C. albicans* infection.

1.2.4 Th17/IL-17R signaling is at the center of antifungal immunity

1.2.4.1 The role of Th17/IL-17 signaling; evidence from human PIDs and murine data

Increased incidence of oral thrush in HIV^+ patients and the strong correlation to low CD4 counts opened up investigations into T helper cell lineages and their associated cytokines in antifungal immunity [4]. Initial studies conducted before the discovery of Th17 cells had led to the conclusion that Th1 and Th2 lineages were the mediators of antifungal immunity [89, 90]. Studies with athymic mice further supported the role of T cell mediated immunity in OPC [91]. However, depletion of CD4 or CD8 T cells in a murine OPC model did not exacerbate susceptibility to OPC [92] although adoptive transfer of *Candida*-specific T cells conferred protection against disseminated candidiasis [93]. These studies indicated the role of both innate and adaptive T cells in oral antifungal immunity, but the specific Th cell lineage and associated cytokines were not convincingly defined.

The observation that athymic mice with low T cell numbers succumbed to oral *C. albicans* challenge supported the role of T cell-mediated immunity in OPC [91]. Studies using *Il12p40*$^{-/-}$ (a common subunit shared with *Il23p19* to form functional IL-23 and with *Il12p35* to form functional IL-12) mice led to the interpretation that IFNγ-producing Th1 cells were the primary mediators of oral antifungal immunity. Inconsistent with this idea, however, was the observation that *IFNγ*$^{-/-}$ mice were resistant to OPC [94]. This conundrum was resolved when it was shown that *Il23p19*$^{-/-}$

but not *Il12p35*$^{-/-}$ are susceptible to OPC, thereby demonstrating that the increased fungal burden in *Il12p40*$^{-/-}$ was due to the loss of IL-23 rather than IL-12 [74, 95]. Additional studies with mice lacking IL-17RA or IL-17RC demonstrated increased susceptibility to OPC, thereby shifting the dominant paradigm from a Th1-dominant response to a Th17-dominated response [74, 95, 96]. Although IL-23 is not required for Th17 differentiation, it is required for maintaining effector Th17 functions, and IL-23-deficient mice are susceptible to OPC [74, 97]. Consistent with the protective role of the Th17/IL-17R axis in oral antifungal immunity, onset of AIDS in HIV^+ patients is characterized by a sharp decline in CD4 T cells and a concomitant increase in oral thrush incidence. Early depletion of Th17 cells observed in HIV and SIV (simian immunodeficiency virus) studies and the incidence of oral thrush further added to the evidence supporting the vital role of Th17 cells in OPC [98]. Intriguingly, the decreased CD4 count is neither associated with incidence of invasive candidiasis in HIV^+/AIDS patients nor VVC in HIV^+/AIDS women [99]. Together, these findings highlight the prominent roles of IL-17 in antifungal immunity with particular beneficial impact in the oral cavity.

Findings from human primary immunodeficiency syndromes (PIDs) confirmed the paradigm regarding a vital protective role of the IL-17/T_H17 axis in oral antifungal immunity [3, 100]. Job's syndrome/ autosomal dominant hyper immunoglobulin E syndrome patients (AD-HIES) are characterized by mucocutaneous candidiasis, pulmonary infections, staphylococcal abscesses, eczema and abnormalities in bone and connective tissue. These patients possess mutations in the DNA binding- or SH2 domains of *STAT3*, which lead to decreased retinoic-related orphan receptor *(ROR)-γ* expression (the Th17-specific "master" transcriptional regulator) and decreased frequencies of Th17 cells [101, 102]. Other HIES patients with similar phenotypes were also identified with mutations in *TYK2*, which encodes a kinase important for activation of STAT3

and other STATs [103]. Gain-of-function mutations in STAT1 also predispose patients to autosomal dominant chronic mucocutaneous candidiasis (AD CMC) linked to impaired Th17 immunity. This PID increases STAT1-dependent cellular responses due to delayed phosphoSTAT1 (pSTAT1) inactivation, even in the presence of STAT3-activating Th17 polarizing cytokines. Impaired STAT3 activation ultimately is associated with decreased IL-17A-, IL-17F- and IL-22-producing Th17 cell frequencies [104, 105]. Similarly, autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED, also known as autoimmune polyendocrine syndrome 1, APS1) patients carry *AIRE* mutations which impair central tolerance. These patients are characterized by multiple autoimmune endocrinopathies, which are often preceded by CMC. The latter is attributed to the increase in circulating autoantibodies against IL-17A, IL-17F and IL-22 [106-108]. Further direct evidence came from discoveries of human loss of function mutations in *IL17RA, IL17RC, IL17F, ACT1, RORC, CARD9 and CLEC7A* (encoding Dectin-1), which predispose patients to CMC and/or disseminated candidiasis [109-112]. Thus far, the overwhelming majority of murine data and data from humans with the described PIDs have supported the role of Th17/IL-17 signaling in oral antifungal immunity.

1.2.4.2 IL-17 family; cellular sources and induction requirements in OPC

The IL-17 family of cytokines consists of six structurally related members, namely IL-17A, IL17B, IL-17C, IL-17D, IL-17E (IL-25) and IL-17F. Of these, IL-17A and IL-17F have the closest homology (~60%) and are nearly always produced together. Unlike the other members of the family, IL-17A and IL-17F are encoded by closely linked genes and exist as homodimers and or heterodimers with different binding affinities to their cognate receptor [IL-17A/A (IL-17A) > IL-17A/F (IL-17AF)> IL-17F/F (IL-17F)]. IL-17A is the best characterized member of the family and is often produced by Th17 subset of $CD4^+$ T cells, although IL-17A-producing $CD8^+$ T cells (Tc17)

have also been described. IL-17 is also produced by several innate immune cells including innate-acting TCRβ^{+} cells (natural Th17 cells, "nTh17"), γδT cells, group 3 innate lymphoid cells (ILC3s), natural killer T (NKT) cells [86, 113, 114]. Although controversial, some myeloid cells and B cells have also been shown to produce IL-17 [115]. These innate and adaptive sources of IL-17 are collectively called Type 17 cells. Type 17 cells also produce other cytokines such as IL-22, and GM-CSF (granulocyte-macrophage colony-stimulating factor).

In the context of acute OPC, nTh17 cells undergo proliferative expansion and are the largest source of IL-17, followed by γδT cells [37, 86]. This is in contrast to a skin *C. albicans* infection model where γδT cells instead are the major source of IL-17 [116]. One other group has reported IL-17 expression from ILC3s in OPC, but this appears to be a minor population [117]. RORγt is the lineage-defining 'master transcription factor' for Type 17 cells and works in conjunction with STAT3 to promote Th17 differentiation and effector functions. RORγt deficiency impairs IL-17 production in all Type 17 cells, and *Rorc*$^{-/-}$ mice are susceptible to OPC [86]. In contrast, STAT3 is not required in CD4 T cells for IL-17-mediated immunity to acute OPC [86] but is required for conventional Th17 cells. Type 17 cells also express the chemokine receptor CCR6, which binds to the mucosal ligand CCL20. The CCR6/CCL20 axis is therefore thought to promote recruitment of Type 17 cells to the target mucosal tissue in response to insults and explains the enrichment of Type 17 cells in mucosal tissues. However, CCR6 is dispensable for expansion of nTh17 cells in acute OPC, and *CCR6*$^{-/-}$ mice are resistant to OPC [37]. This could mean that CCR6 may instead be important in conventional Th17 cells in adaptive immune settings but this has not been proven experimentally.

Generally, IL-17 expression in Type 17 cells during OPC is dictated by Th17 polarizing cytokines IL-23 and IL-1, which are released in response to activation of host PRRs by pathogen-

derived PAMPS. Unexpectedly, prototypical fungal PRRs such as Dectin1 or TLR2, which are important in driving conventional Th17 polarization, were dispensable for innate *Il17a* induction and expansion of nTh17 cells [37, 42]. Instead, *Il17a* induction and nTh17 expansion in acute OPC required the *C. albicans* toxin candidalysin [34, 37]. In contrast, while the CLR downstream adaptor CARD9 is not required for protection against acute OPC, it is protective in T cell recall OPC settings and required for efficient IL-17A expression. Thus, there appear to be specific requirements for *Il17a* induction and Type17-mediated protection in innate and adaptive OPC settings.

1.2.4.3 IL-17 signal transduction: The case of OPC

The IL-17 family of cytokines signal through 5 receptors, IL-17RA-ILRE. IL-17RA is a common receptor which forms complexes with the other IL-17 receptors to transmit signals from the IL-17 family. Homodimers/heterodimers of IL-17A and IL-17F signal through the IL-17RA-IL-17RC receptor complex [118]. A recent report suggested that IL-17A homodimers in addition may signal through the IL-17RA/IL-17RD complex, at least in keratinocytes [119]. Although not yet determined for *Il17rd*$^{-/-}$ mice, both *Il17ra*$^{-/-}$ and *Il17rc*$^{-/-}$ mice are susceptible to OPC [74, 96]. IL-17R-mediated signaling requires the recruitment of the cytosolic adaptor Act1, which distinguishes the IL-17R pathway from other signaling cascades such as IL-1R/TLR pathways [113, 120, 121]. Most studies in disease settings have demonstrated roles for IL-17RA and Act1 in the non-hematopoietic compartment [3, 122]. Consistent with this, loss of IL-17RA in K13-expressing superficial OECs of the tongue recapitulated the susceptibility of global *Il17ra*$^{-/-}$ mice to OPC [88]. Hence, superficial OECs dominantly control IL-17R-mediated responses against OPC.

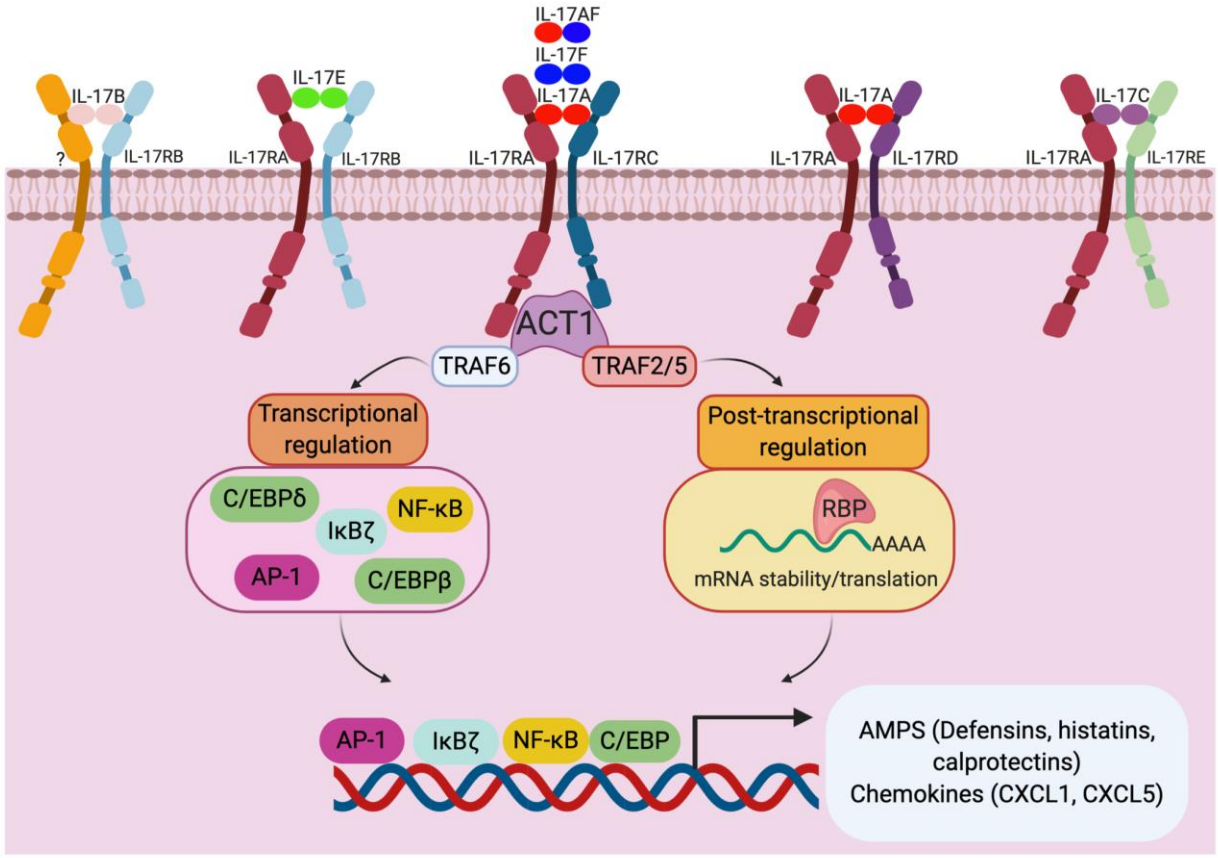

Figure 1.4: IL-17 signaling in OPC

Activation of the IL-17 receptor results in the recruitment of ACT1 and TRAFs to the receptor. TRAF6 activation triggers the induction of transcription factors including NF-κB, IκBζ, C/EBPβ, C/EBPδ and AP-1. The ACT1-TRAF5-TRAF2 complex on the other hand promotes post transcriptional control of IL-17 target genes partly through RNA binding proteins (RBP). In OPC, this IL-17 dependent signaling activates neutrophil recruiting chemokines and antimicrobial peptides (AMPs) to promote fungal clearance.

Upon engaging its receptors (**Fig. 1.4**), IL-17 exerts two broad signaling events; *de novo* transcription and or post-transcriptional regulation of target mRNAs. New transcription is facilitated by the recruitment of Traf6 to Act1-bound IL-17RA and its subsequent ubiquitination

[113]. These signaling events ultimately promote NF-κB, C/EBPβ, C/EBPδ and MAPK dependent signaling cascades. In IL-17-induced NF-κB signaling, the transcription factor IκBζ (encoded by *Nfkbiz*) is a central regulator of IL-17 target genes, and many IL-17 target genes possess IκBζ binding sites [123-126]. Similar to IκBζ, C/EBPβ and C/EBPδ binding sites are present and required in promoters of some IL-17 target genes. Although less dominant and cell type-dependent, IL-17 also activates the mitogen-activated protein kinase (MAPK) pathways including extracellular signal regulated kinase (ERK1/2), p38 and JUN N-terminal kinase (JNK) [113, 127, 128]. In addition to inducing transcription of target genes, IL-17 modulates target transcripts via post transcriptional events through ACT1-TRAF2-TRAF5 interactions. IL-17 induced post-transcriptional control of target genes is achieved through alterations in mRNA stability and or translation through RNA binding proteins (such as MCPIP1/Regnase-1, ARID5A, HuR), micro RNAs (such as miR-23b, miR30a) and ubiquitin mediated signaling. Together, these events are thought to enhance the profound biological consequences of IL-17 signaling [113].

IL-17 promotes antifungal immunity in OPC through a classic panel of genes including neutrophil recruiting factors such as the CXC chemokines (CXCL1, CXCL2 and CXCL5), G-CSF, myeloid and lymphoid chemoattractants (CCL20 and MCP1) and antimicrobial peptides (β-defensins, lipocalin 2 and S100A8/9) [74, 88]. Although there are varied reports on the role of IL-17 signaling in neutrophil recruitment during OPC [74, 88, 129], *Ccr2*-deficient mice (which lack the common CXC chemokine receptor) or mice in which neutrophils are depleted exhibit exacerbated OPC [130]. Similarly, *Defb1*$^{-/-}$ and *Defb3*$^{-/-}$ mice are also susceptible to OPC, with comparable fungal burdens to *Il17ra*$^{-/-}$ mice [88, 131]. However, CCL20, a lymphoid chemoattractant with antimicrobial properties, is not critical for oral antifungal immunity, as neither depletion of CCL20 nor *Ccl20* deficiency increased susceptibility to OPC [132]. IL-17 is

also thought to act on salivary gland epithelial cells to induce expression of AMPs such as histatins (only in humans) and β–defensins in saliva. Consequently, decreased AMP expression in HIES and xerostomia patients partly accounts for the incidence of oral thrush [133, 134]. Together these findings support a strong role for IL-17 target genes in oral antifungal immunity.

Additionally, other cytokines including IL-36 have been shown to be important in driving oral antifungal immunity [135]. During hyphal invasion, candidalysin-induced epithelial damage promotes the production of IL-1, IL-36 and other cytokines. In turn, IL-1 and IL-36 drive Type 17 immunity and nTh17 proliferation through two parallel pathways; while IL-1 drives IL-17 expression, IL-36 promotes IL-23 expression [37, 136]. Thus, while IL-17 is clearly playing a dominant role, effective antifungal immunity is actually a concerted effort of multiple cytokines and pathways that reduce fungal burdens while limiting exacerbated inflammation.

1.2.5 IL-22/IL-22RA1 signaling axis in OPC

1.2.5.1 IL-22; cellular sources and induction requirements

IL-22 is a member of the IL-20 subfamily of IL-10 family cytokines (consisting of 8 members, namely IL-10, IL-19, IL-20, IL-24, IL-26, IL-28A, IL-28B and IL-29). Like humans, murine *Il19, Il20* and *Il24* are located on chromosome 1q32 [137]. *Il22, Il26* and *IFNγ* are found within the same vicinity on chromosome 12q15 although *Il26* is absent in mouse genome [138]. As a family, these cytokines are classified based on their structural similarity, shared receptors and downstream signaling. IL-10 and IL-22 are the best studied cytokines in this family with about 22% homology in mice and 25% homology in humans, respectively. IL-22 consists of six alpha helices (A-F) and an N terminal helix, which are compacted to form a left-handed helical cytokine.

IL-22 is initially synthesized as a 179 amino acid polypeptide that undergoes proteolytic cleavage and glycosylation to generate a mature 146 amino acid bioactive protein [139, 140].

IL-22 is produced by innate and adaptive immune cells including αβ T cells, γδ T cells, NKT cells and innate lymphoid cells (ILCs). Although less common, some studies have also reported IL-22 expression in neutrophils, macrophages and fibroblasts [141, 142]. The dominance of these cellular sources of IL-22 are context-dependent. In addition to their primary cytokines, IL-22 expression has been reported in Th1 and Th17 cells. Additionally, a lineage of αβ CD4 T cells that produce only IL-22 has been described, sometimes called Th22. In mice, however, T cell-derived IL-22 is mostly ascribed to Th17 cells [143-146]. Similar to CD4 T cells, IL-22 production has been identified in a subset of CD8 T cells that produce IL-22 alone or both IL-17 and IL-22 [147-150]. In the context of fungal infections, *C. albicans*-specific IL-22-producing Th17 cells and Th22 cells have been reported in human peripheral blood mononuclear cells (PBMC), and are thought to be more abundant than *C. albicans*-specific IL-17 producing Th17 cells [151]. Additionally, γδ T cells also produce a significant amount of IL-22. While IL-17-producing γδ T cells have been reported in murine OPC [86], and are expanded in HIV^+ patients [152], there are few reports of IL-22 expressing γδ T cells in the context of OPC.

Another principal source of IL-22 are ILCs. ILCs are a heterogenous population of cells lacking rearranged T cell and B cell receptors. Similar to Th1, Th2 and Th17 subsets, ILCs are classified as ILC1, ILC2 and ILC3 based on their expression of lineage defining transcription factors and cytokines. Group 3 ILCs (ILC3) produce IL-17 and IL-22 and can be further broadly classified based on their expression of natural killer cell cytotoxicity receptors (NCRs) such as Nkp44 in humans [153] and Nkp46 in mice [154, 155]. Like Th22 cells, a subset of ILC3s (initially named NK22) cells have been described which only produce IL-22 [153, 156]. While IL-17-

expressing ILC3s have been described in OPC [117], it is not known whether these cells produce IL-22. IL-22 producing NKT cells have also been described [157, 158] but there is not yet any report of IL-17 or IL-22 producing NKT cells during OPC.

Optimal production of IL-22 is dependent on a number of polarizing cytokines and transcription factors. Similar to IL-17-producing Type 17 cells, IL-23 signals on ILC3s, γδ T cells and NKT cells to promote IL-22 expression [146, 159-161]. Not surprisingly, *Il22* deficiency mirrors *Il23* deficiency in many settings [74, 162, 163]. Dendritic cells are a major source of IL-23. Through TLR2, TLR4, TLR5, TLR7, TLR8 and Dectin1/2 activation via their respective PAMPS, Th17 polarizing cytokines (IL-1β, IL-21, IL-6, TGF- β and IL-23) are secreted from dendritic cells [142, 164, 165]. IL-23 promotes STAT3 activation and subsequent upregulation of RORγT in Type 17 cells to induce not only IL-17A and IL-17F but also IL-22 [146, 161, 166, 167]. In the skin, nociceptive nerves promote IL-23 production from dermal dendritic cells to drive Type 17 cell polarization [116, 168]. A subset of nociceptive nerves ($TRPV1^+$ neurons) was recently shown to be sufficient to initiate Type 17 inflammation [169]. IL-1β from multiple cell types also drives IL-22 production independently of IL-23, which can synergize to further increase IL-22 output [170, 171]. Additionally, the aryl hydrocarbon receptor (AhR) directly regulates *Il22* expression and Th17 development in the presence of AhR ligands, at least in the gut. AhR ligands include dietary or microbiota derived products such as indol-3-carbinol, 6-formylindolo(3,2-b) carbazole (FICZ) as well as factors such as physical shear stress, cyclic AMP and Ca^{2+} [142, 172, 173]. It is therefore evident that, while IL-17 and IL-22 share many similar sources and induction requirements, the dominant cellular sources are context-dependent.

1.2.5.2 IL-22 receptor signaling

Members of the IL-10 family signal through heterodimeric receptors consisting of α and β chains. While the α–chains (IL-10RA, IL-20RA, IL-22RA1) have higher ligand binding affinity, the β chains (IL-10R2 and IL-20R2) have lower binding affinity and often undergo a two-step binding process with their respective cytokines. The initial ligation to the α–chain creates a high affinity complex which subsequently binds to the corresponding β–chain. Ligand specificity and cellular response is therefore defined by the expression of the α–chains [174-177]. IL-10 signals through IL-10RA and IL-10RB receptor complex (**Fig. 1.5**) while IL-19, IL-20 and IL-24 signal through IL-20RA and IL-20RB receptor complex [178, 179]. In addition, IL-20 and IL-24 but not IL-19 are able to signal through the IL-22RA1 and IL-20RB receptor complex. IL-26 signals through the IL10RB-IL20RA heterodimer, while IL-28A, IL28B and IL-29 signal through the IL-10RB-IL-28 receptor. In contrast, IL-22 signals through a complex consisting of IL-22RA1 and IL-10RB. While IL-10RB is ubiquitously expressed, IL-22RA1 is mostly expressed in non-hematopoietic cells, thus restricting its functional roles mostly to the stromal compartment [139, 142]. Recent studies, however, have indicated IL-22RA1 expression in lung macrophages during *K. pneumonia* and *M. tuberculosis* infection [180, 181]. Thus, although the majority of available data support the roles of IL-22RA1 in non-hematopoietic cells, it is likely that perturbations in specific disease contexts could change receptor localization patterns, a subject that needs further investigation.

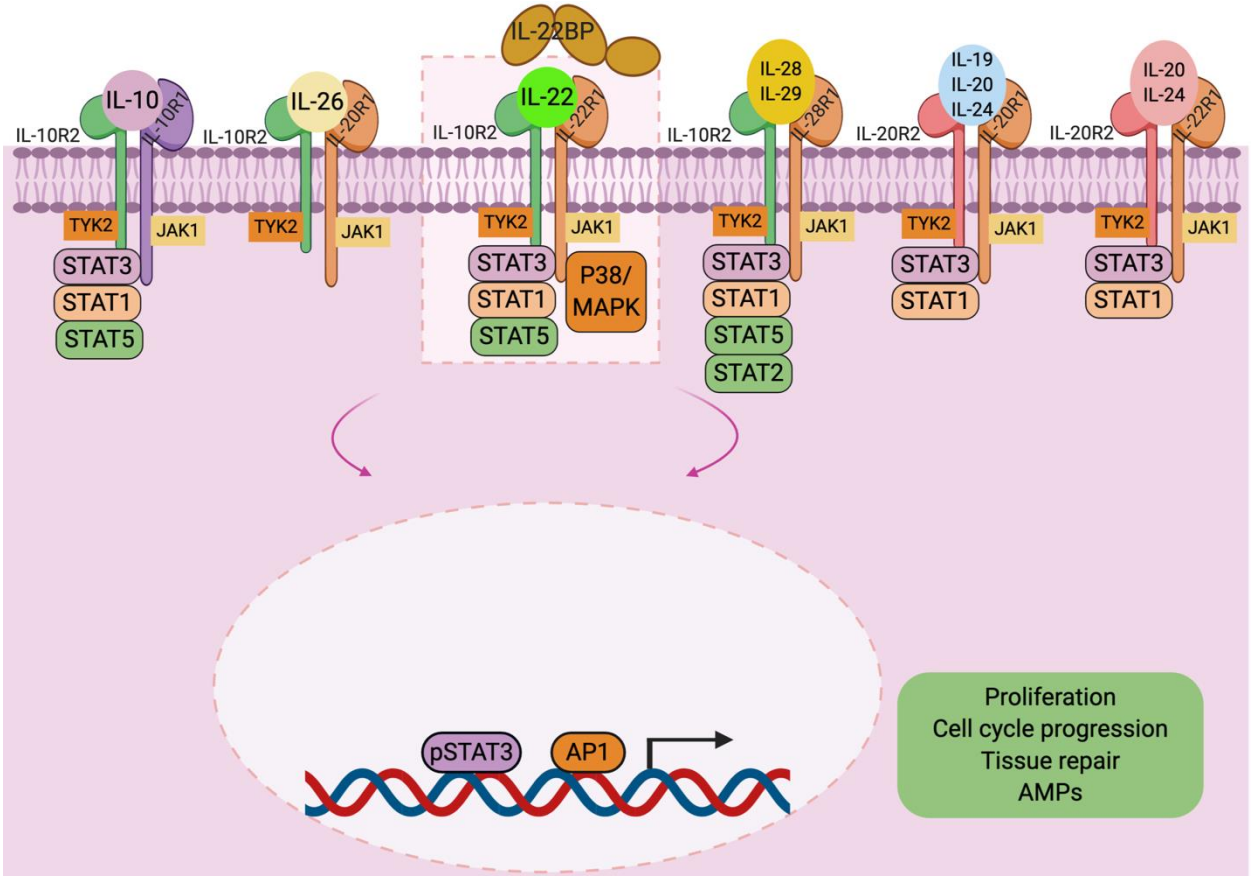

Figure 1.5: IL-10 family cytokines, receptors and signaling

IL-22 is a principal member of the IL-20 subfamily of IL-10 family cytokines. IL-22 signals through the receptor complex consisting of IL-22R1 and IL-10R2. IL-22BP (IL-22R2) is a soluble IL-22R1 decoy receptor which negatively regulates IL-22 signaling. IL-10R2 is shared with the receptors for IL-10, IL-26, IL-28 and IL-29. IL-22R1 can also pair with IL-20R2 to form the receptor complex for IL-20 and IL-24. Additionally, IL-20, IL-24 and IL-19 signal through IL-20R1- IL-20R2 complex. IL-22 signaling activates STAT3 as well as STAT1, STAT5 and p38/MAPK signaling in other contexts to drive genes important for proliferation, tissue repair, cell cycle progression, antimicrobial peptides etc.

Upon binding to the IL-22 receptor complex, the receptor-associated tyrosine kinases Jak1 and Tyk2 are cross-phosphorylated and activated. In turn, activated Jak1/Tyk2 phosphorylate STATs. Although STAT3 is the primary mediator of IL-22 signaling, STAT1 and STAT5 phosphorylation have been observed in response to IL-22 stimulation [182, 183]. Like the other STATs, STAT3 phosphorylation at the tyrosine residue at position 705 dictates its dimerization, nuclear translocation and transcriptional activity. Serine 727 phosphorylation on the other hand enhances STAT3 transactivation and drives non-canonical transcriptional functions of STAT3 in the mitochondrion and endoplasmic reticulum [182, 184]. Beside STATs activation, IL-22 also activates the MAP kinase and p38 pathways [185-188].

1.2.5.3 Regulation of IL-22 signaling

IL-22 signaling is regulated at multiple levels. At the level of the receptor, GSK3β phosphorylates Serine 410 and 414 on IL-22RA1 to prevent proteasomal degradation. The resulting enhanced stability tends to promote IL-22 signaling [189]. IL-22 also binds to a soluble IL-22 receptor subunit called IL-22 binding protein (IL-22BP) or IL-22RA2. IL22BP shares overlapping binding sites with IL-22RA1 and binds to IL-22 with affinity about 1000-fold higher than the membrane bound IL-22RA1. IL-22BP therefore competes with IL-22RA1 to bind to IL-22 and functions as a negative regulator of IL-22 signaling [142, 180, 190]. Similarly, suppressor of cytokine signaling (SOCS) proteins serve as key regulators of IL-22/JAK/STAT pathway by negatively regulating STAT signaling. SOCs 1-3 and cytokine inducible SH2-containing-1 (CIS-1) are the best characterized members of the family. While CIS-1 negatively regulates JAK2/STAT5 pathway, SOCS1 and 3 negatively regulate STAT3 signaling. SOCS1-3 and CIS proteins are therefore aberrantly expressed in a number of inflammatory conditions and cancer settings where their activities serve to potentiate JAK/STAT signaling [191, 192]. Protein

inhibitors of activated STATs (PIAS) family proteins also negatively regulate STAT dependent transcription. While PIAS3 negatively regulates STAT3 driven transcriptional activity, PIAS1 together with other transcriptional repressors block STAT1 signaling by limiting binding to target DNA sequences [193, 194]. Additionally, other factors including activating transcription factor 3 (ATF3) have emerged as positive regulators of IL-22 signaling in inflammatory bowel disease (IBD). IL-22 induced ATF3 regulates STAT3 signaling by negatively targeting protein tyrosine phosphatases (PTPs) such as SHP2 and PTP-Meg2 which are needed to dephosphorylate activated STAT3 into inactive STAT3 [195]. Together, these reports reveal a tightly regulated program to control IL-22 signaling both upstream and downstream of IL-22RA1. Consequently, failure to enforce this regulatory balance could have dire consequences.

1.2.5.4 IL-22 signaling in pathophysiology and tissue regeneration

Studies in liver, lung, skin, thymus, pancreas, kidney, gastrointestinal tract, heart, adipose tissue, breast and eye suggest that IL-22 exerts complex, context-dependent effects under pathophysiological conditions [6, 142, 196]. Generally, IL-22-mediated protection against hepatitis, pancreatitis, colitis, genotoxic stress and thymic injury are attributable to its tissue repair and regenerative properties on target tissues [140, 197, 198]. This repair process is often driven through healthy tissue proliferation and synthesis of antimicrobial peptides. However, in other settings with chronic IL-22 exposure such as psoriasis, IL-22 drives hyperproliferation and inflammation through the expression of proinflammatory mediators such as IL-1, IL-6, G-CSF and GM-CSF [139, 197, 199]. Fine balance is therefore key to determine the physiological outcome of IL-22, whether protective or pathological. The context-dependent role of IL-22 is also determined by other cytokines in the tissue microenvironment. Type 1 interferons tend to shift IL-22-mediated STAT3 signals to STAT1 responses [200]. Other cytokines including IL-17, IL-19, TNF-α, IL-

1β and IL-36 exert additive or synergistic responses with IL-22 [201-203]. Hence, the physiological outcomes of IL-22 are largely influenced by the specific tissue microenvironments and the contributions of other available cytokines and regulatory factors.

The physiological outcomes of IL-22 are highlighted by its roles in different tissues and disease contexts. These outcomes help to provide clearer understanding of the underlying mechanisms. In human gingival fibroblasts, IL-22 cooperates with IL-1β to induce CCL20 expression and subsequent Th17 recruitment to promote periodontal disease [204]. In a psoriatic skin inflammatory model, combinations of IL-17A, IL-22, IL-1α, OSM and TNF-α recapitulated features of psoriasis, including increased chemokine and AMP production [205]. IL-22 also promotes keratinocyte proliferation, migration and tissue remodeling. During epithelial repair, IL-22-induced matrix metalloproteinases (MMP1 and MMP3) are important for epithelial migration [188]. In addition, IL-22 limits keratinocyte differentiation and maturation by inhibiting expression of differentiation markers such as keratins (KRT1, KRT10), profilaggrin, involucrin, loricin, kallikrein 7 and desmocollin [142, 188, 206]. Similar to the skin, IL-22 acts on the lung epithelium to induce AMPs (such as lipocalin 2 and β defensin 2) and chemokines, promote proliferation and repair of the lung epithelium after injury [159]. These activities of IL-22 on the lung epithelium are important in driving protection against *Streptococcus pneumoniae* and *Klebsiella pneumonia* infections [159, 207].

IL-22 can however exert opposing functions even with infections with the same pathogen, and other facets of IL-22 mediated mechanisms are beginning to emerge. While IL-22 is protective against acute *Aspergillus fumigatus* lung infection [208], IL-22 drives lung pathology during chronic exposure [209]. In a bleomycin-induced lung injury model, IL-22 ameliorated disease

severity only in the absence of IL-17A, because the presence of IL-17A exacerbated airway inflammation [210, 211].

In addition to driving changes in epithelial cells/keratinocytes, IL-22 targets stem cells. IL-22 acts on hepatocytes and liver stem cells to induce proliferation and regeneration through the induction of proteins such as Bcl-2, Bcl-X_L, cyclin d-dependent kinase 4 (CDK4), cyclin D1, c-myc and p2 [212-215]. Evidence of IL-22 mediated protection also exists for the gastrointestinal tract where it is important for gut inflammatory disorders, antimicrobial immunity, and cancer immunity [142, 153]. A recent publication showed that in response to genotoxic stress, IL-22 promotes DNA damage response in intestinal stem cells, which is important for reducing the incidence of colon cancer [216]. In addition to the classical roles of IL-22 in mucosal epithelia, IL-22 also acts on the stromal environment in secondary and tertiary lymphoid tissues to control lymphocyte effector functions. During thymic injury, IL-22 promotes the regeneration of thymic epithelial cells to restore T cell function [140]. Similarly, IL-22 is thought to act on spleen and thymic stromal cells to restrain T cell activation during acute viral infection [217]. In line with this activity, IL-22 also acts on keratin 14 expressing cells of the skin to regulate MHCII expression and ultimately influence the responses to *Staphylococcus* epidermidis infection [218]. Collectively, these reports indicate that IL-22 does not only act on stromal cells of mucosal barriers to drive antimicrobial immunity and promote mucosal integrity but can also indirectly dictate the outcome of lymphocyte effector responses.

1.3 Summary

Physiological outcomes of inflammation, injury and infections are dictated by the collective contributions of multiple factors; the secreted cytokines, the responding cells, as well as efficient regulatory mechanisms [219]. Thus, while some mediators may have more dominant roles, the overall outcomes are determined by the interplay of these multiple factors rather than a single cytokine, cell type or regulatory mechanism. This chapter discusses the current state of our understanding of oral antifungal immunity mainly from the perspectives of IL-17, IL-22, the oral mucosa and the underlying mechanisms at play.

Both IL-17 and IL-22 are implicated in antifungal immunity and are thought to mainly act on non-hematopoietic cells. In view of their dominance in OPC settings, it is critical to understand their specificity and how they collectively work in concert to integrate multiple signals for the benefit of the host. These advancements will shed better light on the oral mucosa and immunity in the oral cavity particularly with respect to IL-22.

2.0 Materials and Methods

2.1 Materials

2.1.1 Mice

Il22TdTomato, Il22ra1$^{fl/fl}$, *K13*^{Cre}, *Card9*$^{-/-}$, *K14*^{CreERT2}, *STAT3*$^{fl/fl}$, *Saa1/2, Il23r*$^{-/-}$ mice and *Act1*$^{-/-}$ were described [88, 220-224]. *Il17ra*$^{-/-}$ mice were from Amgen and *Il22*$^{-/-}$ mice were from Genentech. All other mice were from JAX or Taconic Farms. Mice were on the C57BL/6 background and housed in specific pathogen free (SPF) conditions. Experiments were performed on age-matched mice (6-10 weeks) of both sexes. Experiments were performed in accordance with protocols approved by the University of Pittsburgh IACUC and NIAID and followed guidelines in the Guide for the Care and Use of Laboratory Animals of the NIH.

2.1.2 Cells and Cytokines

Human TR146 OECs (ECAAC10032305) were cultured in Dulbecco's Modified Eagle Medium (DMEM)–F12/15% fetal bovine serum as previously described [84]. Recombinant human IL-22 (PeproTech 200-22) was used at 100ng/ml.

2.1.3 Antibodies

Antibodies used are listed in Table 2.1

Table 2.1: List of antibodies

Antibody	Vendor	Purpose	Catalog #	Dilution
STAT3	Cell signaling	Western blot	9139	1:1000
pSTAT3	Cell signaling	Western blot IF	9145	1:1000 1:100
c-Fos	Cell signaling	Western blot	2250	1:1000
pC-Fos	Cell signaling	Western blot/ IF	5348	1:1000
IκBζ	Cell signaling	Western blot	9244	1:1000
pP65	Cell signaling	Western blot	3033	1:1000
P65	Cell signaling	Western blot	8242	1:1000
ERK	Cell signaling	Western blot	4695	1:1000
pERK	Cell signaling	Western blot	9106	1:1000
JNK	Cell signaling	Western blot	9252	1:1000
pJNK	Cell signaling	Western blot	4668	1:1000
β-actin-HRP	Abcam	Western blot	Ab49900	1:25000
Keratin 14	Abcam Abcam Invitrogen	IF Flow cytometry IF	Ab181595 Ab210414 MA5-11599	1:300 1:5000
Keratin 13	Abcam	IF	Ab92251	1:300
IL-22RA1	R&D	IF	MAB42941	1:100

Table 2.1 continued

Antibody	Vendor	Purpose	Catalog #	Dilution
CD45 Pac Blue, AF 700	Biolegend	Flow cytometry	103125 103128	1:100
CD45.1 FITC	Biolegend	Flow cytometry	110705	1:100
CD45.2 PE	Biolegend	Flow cytometry	109807	1:100
CD44 eFluor 450	eBioscience	Flow cytometry	48044182	1:100
CD4 APC Fire 750, Qdot 605	Biolegend ThermoFisher	Flow cytometry	100459 Q10008	1:100 1:500
CD11B AF 700	Biolegend	Flow cytometry	101222	1:100
Ly6G PE	BD	Flow cytometry	551461	1:100
TCRβ FITC, PE, PerCP-Cy5.5	eBioscience Biolegend BD	Flow cytometry	11596185 109207 560657	1:100
TCRγδ eFluor 450, PE	Invitrogen eBioscience	Flow cytometry	48571182 125781181	1:100
EpCAM FITC		Flow cytometry	11579182	1:100
IL-17A APC, PE	Invitrogen BD	Flow cytometry	11717781 559502	1:100
IFN-λ PE-CF594, APC	BD	Flow cytometry	562303 554413	1:100

2.1.4 qRT-PCR primers

Primers for qRT-PCR were purchased from Qiagen and are listed in Table 2.2

Table 2.2: List of commercial qRT-PCR primers

Gene Symbol	Organism	Catalogue #
Il17a	Mouse	QT00103278
Il17f	Mouse	QT00144347
Il22	Mouse	QT00128324
Il17ra	Mouse	QT00112063
Defb3	Mouse	QT00265517

2.2 Methods

2.2.1 Primary and Recall OPC Model

C. albicans (CAF2-1, SC5314 and strains) were grown overnight for 16 hours in yeast extract peptone dextrose (YPD) broth at 30ºC with continuous agitation. *C. albicans* suspension was prepared to a final density of $2x10^7$ cells/ml. Mice were anesthetized (100mg/kg Ketamine and 10mg/kg Xylazine in 0.9% sterile saline) and sublingually inoculated with pre-weighed (2.5mg) cotton balls saturated with *C. albicans*. Control sham mice were similarly inoculated with pre-weighed cotton balls soaked in PBS. Mice remained under anesthesia for 75 minutes after which the cotton balls were taken out. Mice were also administered 1ml of 0.9% saline before and after oral challenge. Anti-IL-22 Abs (Genentech, clone 8E-11) or control IgG1 (Bio X Cell)

(150ug) were injected i.p. on days -1, 0, 1, 2, 3 relative to infection. Anti-IL-17A or IgG2a control Abs (200ug) were from Janssen Research & Development LLC [225] and administered i.p. on days -1, 1, and 3 relative to infection. For primary OPC, mice were weighed daily until harvest on days 1, 2, 3, 4 or 5 post infection. One longitudinal half of the harvested tongue was collected, flash frozen in liquid nitrogen and stored at -80ºC for RNA extraction. The other half tongue was homogenized by mechanical disruption using a GentleMacs dissociator (Miltenyi Biotec). Tongue homogenates were plated in triplicate on YPD agar plates containing ampicillin, incubated at 30ºC for 48 hours and quantified by estimating colony forming units per gram (CFU/g) of tongue. For the recall infection model, mice were initially infected with *C. albicans* or inoculated with PBS as described above. After 6 weeks, mice were re-infected with *C. albicans* as described above and organs were harvested at days 1, 2 or 3 post infection. One longitudinal half of harvested tongue was homogenized for CFU enumeration and the other half stored for RNA extraction as described above. For histological analysis, longitudinal sections of harvested tongues were embedded in a cast with optimum cutting temperature (OCT) compound and subjected to temperature gradient freezing. The samples were kept frozen at -80ºC until they were sectioned. Cervical lymph nodes (cLN) were also harvested and manually homogenized in RPMI. The homogenate was filtered and the resulting single cell suspension was stained for flow cytometry or co-cultured with heat-killed (HK) *C. albicans* for quantification of secreted cytokine by ELISA. A summary schematic of the primary infection and recall infection is presented in Fig. 2.1.

2.2.2 Bone marrow chimera

Recipient mice [WT(CD45.1), *Il22ra1*$^{-/-}$(CD45.2) and *Il22*$^{-/-}$(CD45.2)] were put on antibiotics water (Sulfamethoxazole and Trimethoprim) beginning from day -1. Recipient mice

were irradiated (900rads) on day 0. Femoral/ tibial bone marrow (10^6) from donor mice (WT, *Il22ra1*$^{-/-}$and *Il22*$^{-/-}$) were i.v. injected into recipient mice. Mice were withdrawn from antibiotic water after 10 days. After 6 weeks, successfully reconstituted mice were subjected to OPC as described above

2.2.3 Co-culture of cLN cells with HK *C. albicans* and quantification of secreted cytokines

HK *C. albicans* (CAF2-1) extract was prepared by boiling 1ml of (~ $4x10^8$ cells) overnight cultured *C. albicans* for 45 mins at 56ºC. CLN cells were plated at 10^6 cells/well in flat bottom 24-well plates with or without $2x10^6$ HK *C. albicans* in serum-free AIM V media (GIBCO, Invitrogen) containing 1X β-Mercaptoethanol (1000X, GIBCO Invitrogen) and 20U/ml of IL-2. Co-cultures were incubated at 37ºC and 5% CO_2 saturation for 5 days. Culture supernatants were harvested on day 5 and analyzed for secreted IL-17A and IL-22 by ELISA according to the manufacturer's instructions (eBioscience).

2.2.4 Flow cytometry

Flow cytometry of tongue homogenates was performed as described [86]. Tongues were digested with DNase1 (1mg/ml, Roche), and Collagenase IV (0.7mg/ml) in HBSS at 37°C for 30 minutes. Filtered cell suspensions were either stained directly or separated by Percoll gradient centrifugation. Antibodies were from eBioscience, BD Biosciences, BioLegend or Abcam and are listed in Table 2.1. Proliferation was assessed using the Foxp3/Transcription factor Buffer kit (eBioscience) with Ki67-APC/PerCP. To assess apoptosis, $CD45^-EPCAM^+$ cells were stained with caspase-3 apoptosis kit (BD Biosciences) according to the manufacturer's instructions. For cell

cycle analysis, mice were intraperitoneally injected with 1 mg bromodeoxyuridine (Abcam, BrdU flow kit, BD Biosciences) on day 1 p.i. and tongues harvested 24 h later. Harvested tongues were digested as described above and the total single cell suspension was processed for BrdU analysis based on the instructions from the manufacturer. For assessment of intracellular cytokines after recall infection, cLN were harvested on days 2 or 3 p.i. . Single cell suspensions were prepared as described previously and stimulated with 50ng/ml phorbol 12-myristate 13-acetate (PMA) and 500ng/ml ionomycin (Sigma-Aldrich) in the presence of Golgi plug (BD Biosciences) for 4 hours at 37ºC and 5% CO_2 saturation. After staining for surface markers (CD45, CD3, CD4, CD44), samples were fixed, permeabilized and stained for IL-17A and IFN-γ. Samples were stained with GhostDye Violet 510 (TONBO Biosciences, CA) to exclude dead cells. Data were acquired on an LSR Fortessa and analyzed with FlowJo (Tree Star).

2.2.5 Histology, immunofluorescence and immunocytochemistry

Cryosections were stained following the Cell Signaling immunofluorescence protocol (https://www.cellsignal.com/contents/resources-protocols/immunofluorescence-general-protocol/if). Briefly, slides were fixed by incubation in ice cold methanol followed by blocking with 5% goat serum (Life technologies #50062Z), 1% bovine serum albumin (BSA)(Sigma Aldrich #A7906) and 0.3% Triton X (Fisher #BP151). Samples were stained overnight with primary antibodies [IL-22RA1, Keratin 13, Keratin 14, Ki67 and anti-p-STAT3 (Tyr705)]. Samples were subsequently incubated with secondary fluorescence antibodies. Nuclei were stained with DAPI and slides were cover slipped. TUNEL staining was performed with an Apoptosis Detection kit (Millipore). Images were acquired on an EVOS FL microscope (Life Technologies). $Tunnel^+$ cells were enumerated from 10 random fields per slide.

2.2.6 qPCR, RNA-Seq and GSEA

Frozen tongue tissue was homogenized in 500µl of Qiazol lysis buffer (Qiagen) using a GentleMACS Dissociator (M-tubes, RNA-02 program, Miltenyi Biotec). Chloroform was added to the lysates and centrifuged. The upper aqueous layer was collected and RNA was subsequently extracted following instructions of the RNAeasy Mini Kit. RNA concentration was assessed using Nanodrop. Equal concentrations of mRNA from samples were reverse transcribed into cDNA using Superscript III First-Strand synthesis system (Invitrogen) by following the manufacturer's protocol. Gene expression analysis by qPCR was done using Perfecta SYBR Green FastMix ROX (Quanta BioSciences) on a 7300 Real-Time PCR System (Applied Biosystems) and normalized to *Gapdh* [37]. Primers were from QuantiTect Primer Assays (Qiagen) and are listed in Table 2.2. For RNASeq, cDNA libraries were prepared from tongue RNA harvested day 1 p.i. (Nextera XT Kit) and RNASeq was performed on the Illumina NextSeq 500 platform by the Health Sciences Sequencing Core at the University of Pittsburgh. Sequencing reads were annotated and aligned to the UCSC mouse reference genome (mm10, GRCm38.75) using HISAT [226]. HISAT alignment files were used to generate read counts for each gene, and determination of differential gene expression was performed using the DE-seq package from Bioconductor [227]. Unbiased hierarchical clustering of differentially expressed genes with $P<0.05$ was calculated using CLC Genomics Workbench and Partekflow software. Relative expression values in heat maps are TPM (Transcripts per kilobase million). Partekflow and GSEA from the Broad Institute were used to calculate enrichment of genes in each set. Frozen tongue tissue was homogenized in 500µl of Qiazol lysis buffer (Qiagen) using a GentleMACS Dissociator (M-tubes, RNA-02 program, Miltenyi Biotec). Chloroform was added to the lysates and centrifuged. The upper aqueous layer was collected and RNA was subsequently extracted following instructions of the RNAeasy Mini

Kit. RNA concentration was assessed using Nanodrop. Equal concentrations of mRNA from samples were reverse transcribed into cDNA using Superscript III First-Strand synthesis system (Invitrogen) by following the manufacturer's protocol. Gene expression analysis by qPCR was done using Perfecta SYBR Green FastMix ROX (Quanta BioSciences) on a 7300 Real-Time PCR System (Applied Biosystems). Primers were purchased from commercial vendors and are listed in Table 2.2

2.2.7 Western blot

TR146 whole cell lysates were prepared with 1% NP40 lysis buffer complemented with protease inhibitors (Roche), sodium orthovanadate and phenylmethylsulphonyl fluoride (PMSF). Samples were gently agitated on a vortex with intermittent cooling on ice and the total cell extract was obtained by centrifuging at 15000g for 10 minutes. Protein concentration was assessed using Pierce BCA Protein assay kit (Thermo Fisher Scientific). Lysates were boiled with 4X sodium dodecyl sulphate (SDS) at 95°C for 5 minutes. 30µg – 50µg of total protein was loaded in 10% homemade polyacrylamide gels.

3.0 Oral Epithelial IL-22 Signaling Licenses IL-17-Mediated Immunity to Oral Mucosal Candidiasis

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"Oral epithelial IL-22/STAT3 signaling licenses IL-17-mediated immunity to oral mucosal candidiasis"

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3.1 Background

Although IL-17 is the eponymous cytokine of Th17 cells and other "Type 17' lymphocytes, IL-22 is also characteristic of these cells [228]. Multiple studies indicate that IL-22, like IL-17, helps control oral candidiasis. For example, OPC occurs in patients with autoimmune polyendocrine syndrome type 1 (APS-1), a congenital autoimmune syndrome caused by mutations in *AIRE* and characterized by circulating auto-antibodies that neutralize not only IL-17A and IL-17F but also IL-22 [106-108, 229]. Mice with IL-22 impairments (via gene deficiency or antibody neutralization) are susceptible to OPC [74, 230, 231], and reduced IL-22 expression is associated with human chronic mucocutaneous candidiasis (CMC) [232]. Nonetheless, relatively little is known about the mechanisms of antifungal immunity mediated by IL-22 in this setting or in other oral diseases.

The oral mucosa provides a vital physical barrier to limit pathogen invasion, yet mechanisms of oral mucosal immunity remain surprisingly under-studied, especially compared to other mucosal tissues such as the lung or gut. The oral mucosa is a stratified non-keratinizing tissue composed of distinct epithelial layers [80, 233]. A proliferative basal epithelial layer (BEL) undergoes a program of differentiation that gives rise to the post-mitotic suprabasal epithelial layer (SEL). This differentiation process maintains the tissue and restores barrier immunity after infection or injury [80, 82, 234]. Each layer is characterized by expression of specific pairs of cytokeratin filaments. Like most stratified epithelia, BEL expresses keratins-5 and -14 (K14, K5). However, the SEL expresses K4 and K13, which have a more restricted expression pattern. IL-17R signaling activity is generally restricted to non-hematopoietic cells [235, 236], and we previously demonstrated that IL-17RA in $K13^{+}$ SEL cells is essential for immunity to OPC [237]. Similar to the IL-17R, the IL-22 receptor is expressed mainly in non-hematopoietic cells and is

implicated in gastric *C. albicans* infections [238], yet remarkably little is known about IL-22 signaling and function in the oral mucosa.

In this chapter, I show that IL-22 functions non-redundantly with IL-17 to limit oral fungal infection. Although expression kinetics are similar between these cytokines, there were unexpected differences in cellular sources, downstream target gene expression and the nature and localization of the essential cytokine-responsive cells within the stratified oral epithelium. IL-22 induces proliferation and survival of BEL cells. Moreover, IL-22-dependent signals are required for renewing the IL-17-responsive SEL. Hence, IL-22/IL-22RA1 signaling 'licenses' the oral IL-17 signaling response, despite acting in a distinct epithelial layer.

3.2 Results

3.2.1 IL-22 protects against OPC

To compare the roles of IL-22 and IL-17 in OPC, we tracked the time course of *Il22* and *Il17a* mRNA expression in the oral mucosa (tongue) of wild-type (WT) mice following sublingual *C. albicans* infection [86, 239]. *Il22* and *Il17a* transcripts were not detectable at baseline but were induced contemporaneously at ~16-24 h post-infection (p.i.). Expression of both peaked at 48 h and returned to undetectable levels by 96 h (**Figure 3.1A-B**). Similar to our previous report, *Il22*$^{-/-}$ mice were susceptible to OPC, with fungal loads consistently averaging ~10^3 CFU/g of tongue tissue at 5 days p.i., whereas WT mice fully cleared the infection by this time point (**Figure 3.1C**) [74]. Fungal loads in *Il22*$^{-/-}$ mice were typically ~1/2 log lower than in mice with IL-17 signaling defects (*Il17ra*$^{-/-}$ or *Act1*$^{-/-}$), also consistent with prior findings (**Figure 3.1C**) [74, 240, 241].

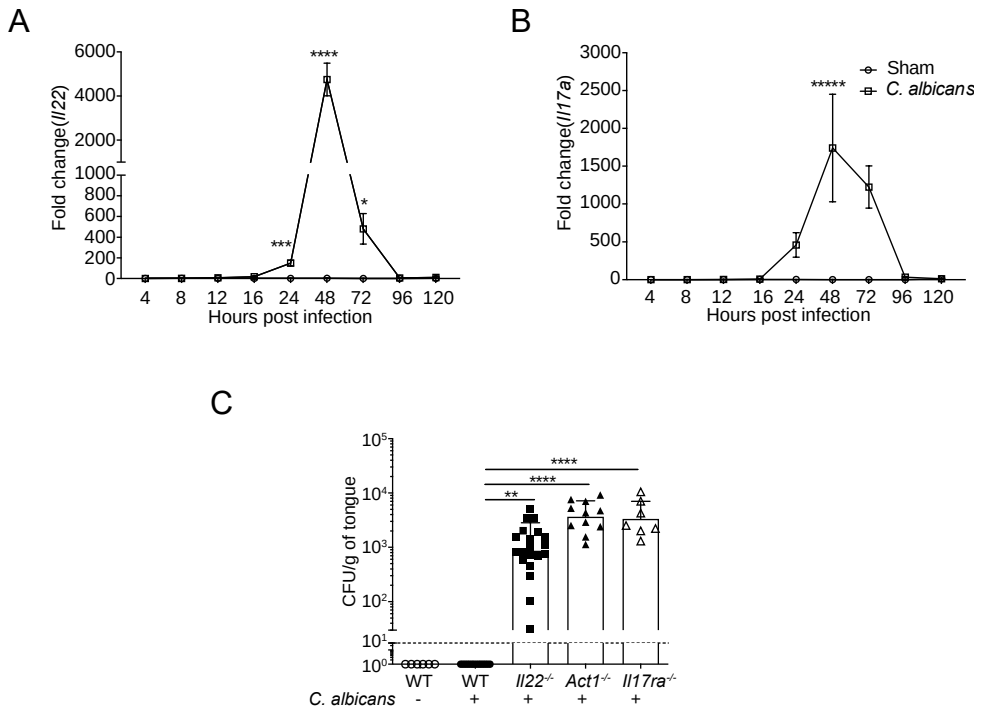

Figure 3.1: IL-22 is protective against OPC

The indicated mice were sublingually inoculated with cotton ball-saturated PBS (Sham) or *C. albicans* (OPC). Each symbol represents 1 mouse. Total mRNA from tongue homogenates of infected WT mice was subjected to qPCR for *Il22* (A) and *Il17a* (B) and normalized to *Gapdh* at each time point. Graphs show mean + SEM. Data are pooled from 4-9 mice per group. (**C)** Fungal burdens were determined by CFU enumeration on YPD/Amp agar at day 5 p.i. Graphs show geometric mean + SD. Data were pooled from 3 independent experiments. Dashed line indicates limit of detection (~30 CFU/g). Data were analyzed by ANOVA or Student's t-test, with Mann-Whitney correction for fungal load analysis. **p<.01, ****p<.0001.

3.2.2 IL-22 is non-redundant with IL-17 receptor signaling in protecting against OPC

We next evaluated the impact of IL-22 on antifungal events in OPC. Since neutrophils are important in antifungal immunity, we assessed neutrophil recruitment by flowcytometry using tongue single cell suspensions. In line with previous data, *Il17ra*$^{-/-}$ mice exhibited impaired neutrophil recruitment to the tongue, measured at day 2 p.i. [74]. Unexpectedly, *Il22*$^{-/-}$ mice showed increased oral neutrophil frequencies and numbers (**Fig. 3.2A, B**), despite there being no differences in fungal load compared to *Il17ra*$^{-/-}$ mice at this time point (**Fig. 3.2** C). *Il17a* and *Il17f* mRNA were elevated in *Il22*$^{-/-}$ mice (**Fig. 3.2 D, E**), which may explain this observation. While IL-22 mostly exerts anti-inflammatory properties, IL-17A and IL-17F tend to be proinflammatory and promote unrestricted neutrophil recruitment. Neutrophil recruitment to the site of infection is a two-edged sword which must be exquisitely fine-tuned. Failure to modulate neutrophil recruitment can promote neutrophil mediated damage to the epithelium. In line with this, a previous study with bleomycin-induced acute lung injury model, showed that IL-22 was only

Because mice are naïve to *C. albicans,* the IL-17 produced during a first encounter derives from innate immune cells [86, 241-244]. We previously showed that antigen-independent CD4^{+}TCR$\alpha\beta^{+}$ cells that express IL-17 ('natural' Th17 cells, nTh17) are required for effective immunity to OPC [37, 86, 245, 246]. These nTh17 cells undergo proliferative expansion during the first two days of *C. albicans* infection in an IL-1R-dependent manner [37, 86, 136]. We therefore sought to determine whether IL-22 contributes to proliferation of nTh17 cells. In *Il22*$^{-/-}$ mice, the frequencies of proliferating (Ki67^{+}) CD4^{+}TCR$\alpha\beta^{+}$ cells were comparable to WT mice following infection (**Fig. 3.2F**), indicating that IL-22 does not regulate nTh17 proliferation.

In view of the many similar functions ascribed to IL-22 and IL-17 at mucosal surfaces

[202], we asked whether IL-22 acts redundantly with IL-17 in OPC. Treatment with neutralizing Abs against IL-22 in *Act1*$^{-/-}$ mice (which are fully impaired for IL-17R signaling [247] resulted in higher fungal burdens than isotype-treated controls (**Fig. 3.3 A**). Similarly, blocking IL-17A in *Il22*$^{-/-}$ mice led to significantly higher fungal burdens compared to isotype-treated controls (**Fig. 3.3B**). Weight loss was more pronounced in mice lacking IL-22 than in WT mice, but addition of anti-IL-17A Abs did not cause further weight loss (**Fig. 3.3C**). Thus, IL-22 and IL-17 act cooperatively but non-redundantly to control OPC.

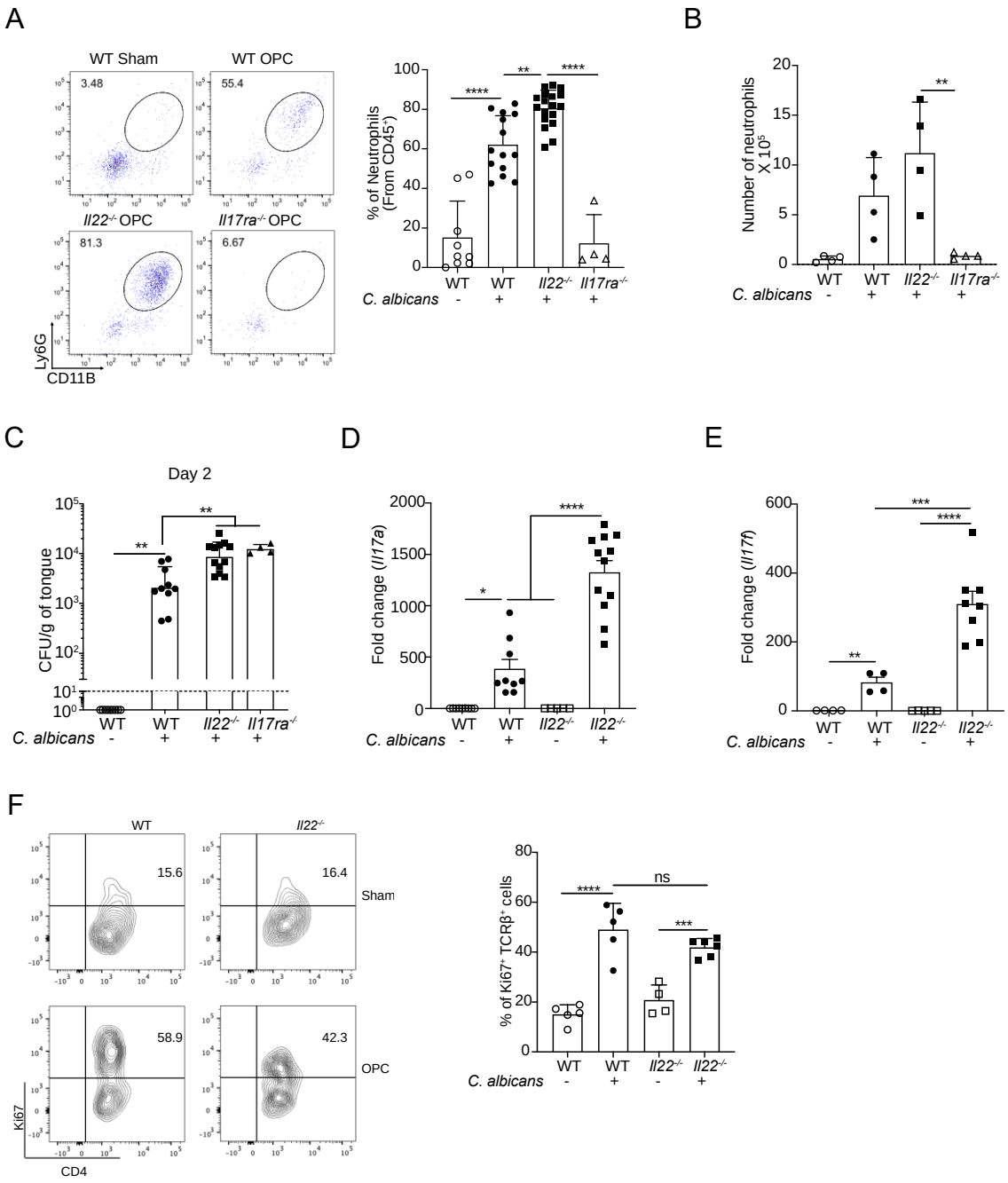

Figure 3.2: IL-22 modulates neutrophil recruitment but not nTh17 proliferation

(A) Tongue homogenates were prepared on day 2 p.i. *Left*: A representative FACS plot showing percent of $CD11b^{+}Ly6G^{+}$ neutrophils (gated on live, $CD45^{+}$ cells). *Right:* Data from 3 independent experiments. (B) FACS plot showing neutrophil numbers from one experiment (C) The indicated mice were subjected to OPC and fungal burdens were assessed at day 2 p.i.. (**D-E).** *Il17a* and *Il17f*

in total tongue RNA was assessed by qPCR relative to *Gapdh* on day 2 p.i.. Graphs show mean + SEM relative to Sham-infected WT mice. (**F)** WT and *Il22*$^{-/-}$ mice were orally challenged with *C. albicans* or PBS. Tongue homogenates were prepared at day 2 p.i. and lymphocytes separated by percol gradient centrifugation. Cells were gated on live lymphocytes. Representative FACS plot of proliferating CD45^{+}TCRB^{+}CD4^{+} (left) and summary plot (right) are shown.

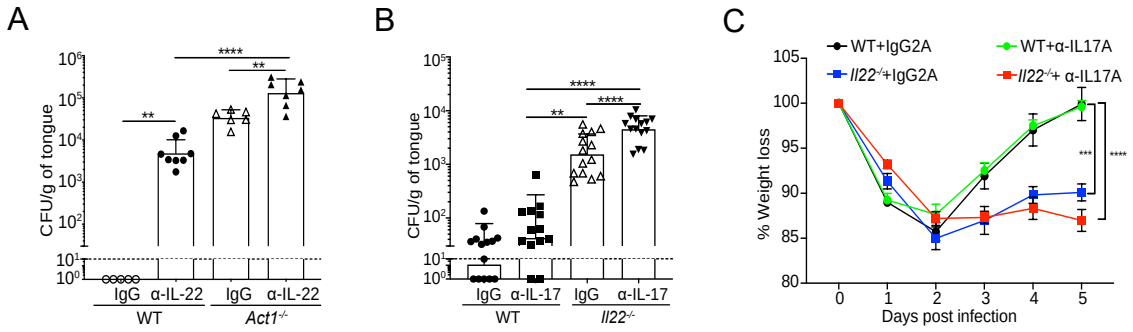

Figure 3.3: IL-22 is non-redundant with IL-17 signaling in oral antifungal immunity

(**A)** Mice were injected i.p. with anti-IL-22 or isotype control IgG (150ug) on days -1, 0, 1, 2, and 3 relative to infection. CFU was assessed on day 4, pooled from 2 independent experiments. (**B).** Mice were treated with anti-IL-17A or isotype control (IgG2a) (200 ug) injected i.p. on days -1, 1, and 3 relative to infection. CFU was assessed on day 5, pooled from 3 independent experiments. (**C)** Weight loss was assessed daily and shown relative to day 0. Data were analyzed by ANOVA or Student's t-test, with Mann-Whitney correction for fungal load analysis. *p<.05, **p<.01, ***p<.001, ****p<.0001.

3.2.3 Determining induction requirements for IL-22 during OPC

Conventional adaptive Th17 responses to *C. albicans* are triggered by sensing of β-glucans in the fungal cell wall via the Dectin-1/CARD9 signaling, which induces Th17-polarizing

cytokines (IL-23 and IL-6) [51, 116, 129, 248-251]. Here, we assessed the essential triggers of IL-22 in innate responses to OPC. IL-23 maintains effector Th17 functions and it is implicated as a critical driver of IL-22 expression in many settings [97, 146, 159, 161, 163]. As expected, *Il23r*$^{-/-}$ mice showed impaired oral *Il22* expression following *C. albicans* infection (**Fig. 3.4A**), consistent with elevated fungal loads seen in *Il23r*$^{-/-}$ mice [74]. However, mice lacking Dectin-1 (*Clec7a*$^{-/-}$) or CARD9 (*Card9*$^{-/-}$) (which are important for β-glucan recognition and signaling downstream of C-type lectin receptors respectively) induced *Il22* mRNA normally (**Fig. 3.4A**). In some settings, IL-22 is induced in Th17 cells by the aryl hydrocarbon receptor (AhR) or serum amyloid A (SAA) [252, 253]. However, *Ahr*$^{-/-}$ mice did not exhibit any obvious deficit in *Il22* induction and were resistant to OPC (**Fig. 3.4B, C**). Similarly, SAA1/2-deficient mice were resistant to OPC and *Il17a*, *Il17f* and *Il22* were induced normally in the oral mucosa (**Fig. 3.4D, E**).

Since these prototypical IL-22-inducing signals were dispensable for acute IL-22 induction, we next evaluated the role of candidalysin, a recently described trigger of anti-*Candida* immunity. This fungal cytolytic peptide, encoded by the *ECE1* gene, is produced only by hyphae and is needed for optimal expression of IL-17 and proliferation of nTh17 cells after oral *C. albicans* challenge [34, 37]. WT mice were infected with *ECE1*-deficient (*ece1Δ/Δ*), candidalysin-deficient (*ClysΔ/Δ)*, or an *ECE1* re-complemented strain (Rev). Infection with *ece1Δ/Δ* or *ClysΔ/Δ* caused reduced *Il22* mRNA expression compared to Rev (**Fig. 3.4F**). IL-22 protein levels were similarly affected, assessed using *IL22TdTomato* reporter mice [Shen et al., 2015] (**Fig. 3.4G**). Notably, these strains showed similar fungal burdens at this time point (**Fig. 3.4H**). Thus, IL-23 and candidalysin but not Dectin-1/CARD9, AhR or SAA1/2 are required for innate IL-22 production during OPC.

3.2.4 γδ-T cells and nTh17 cells are the predominant sources of IL-22 during OPC

We next delineated the oral cellular sources of IL-22 during OPC. As expected, chimeric mice that received *Il22*$^{-/-}$ bone marrow (BM) developed OPC, regardless of recipient genotype, indicating that IL-22 in hematopoietic cells is required for *C. albicans* clearance (**Fig. 3.5A**). We then analyzed tongue tissue from *IL22TdTomato* reporter mice at day 2 p.i., the time point when *Il22* mRNA expression peaks (**Fig 3.1A**). There were almost no detectable CD45^{+}TdTomato^{+} cells prior to infection, indicating that IL-22 is not expressed in the oral mucosa at baseline. After infection however, a substantial population of reporter-positive cells was present (**Fig. 3.5B**). Of these, γδ-T cells constituted the dominant TdTomato^{+} population (58%), followed by CD4^{+}TCRαβ^{+} cells (26%). A population of CD4^{-}TCRαβ^{+} cells (8%) and TCR-negative cells (8%) also expressed the reporter (**Fig 3.5B).** This expression pattern contrasts to some extent with that of IL-17, based on prior studies using an IL-17A fate tracking reporter mouse [37, 86, 254] (**Fig 3.5C**).

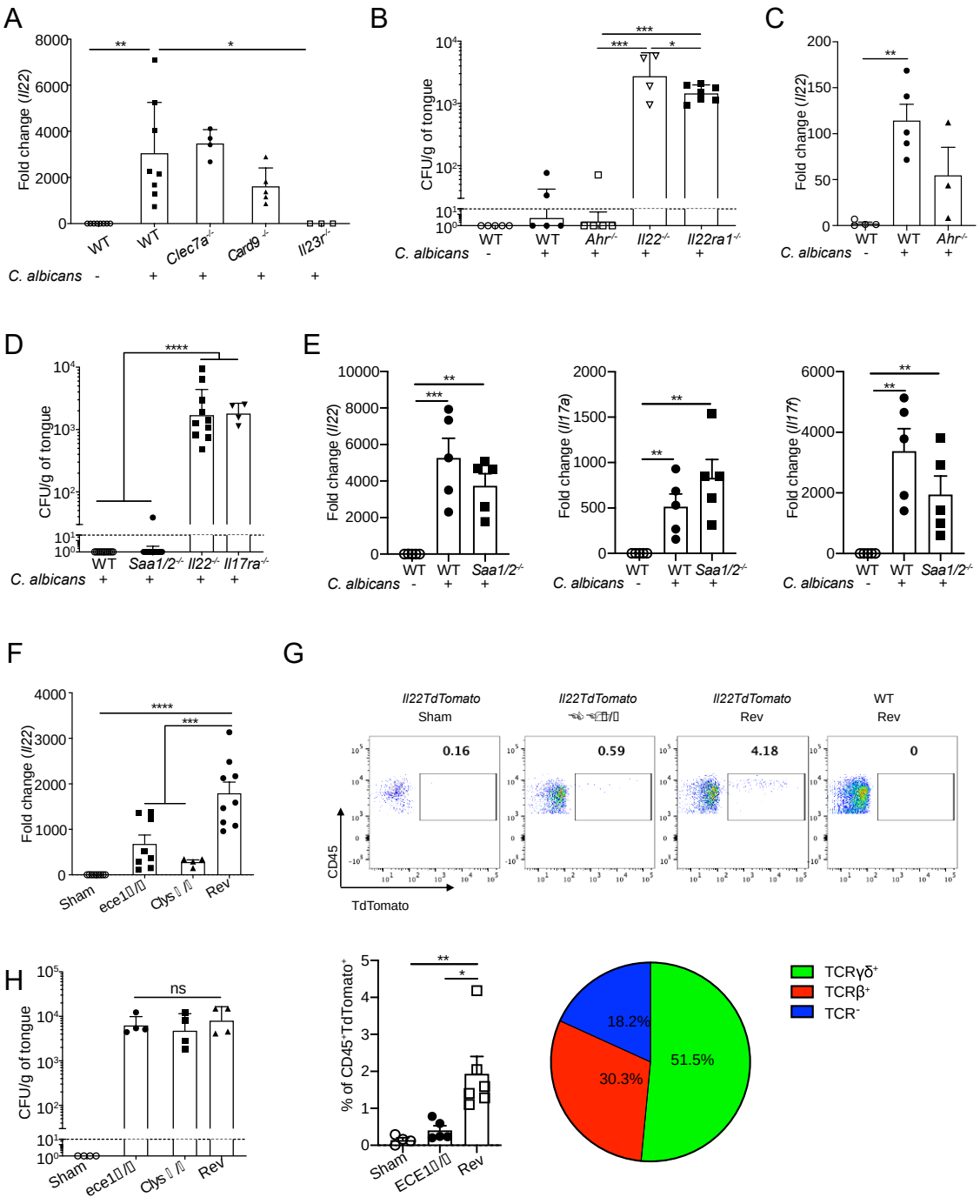

Figure 3.4: Determinants of IL-22 induction in acute OPC

The indicated mice were subjected to OPC. Each symbol represents 1 mouse **(A)** Tongues were harvested on day 2 p.i. and *Il22* mRNA was assessed by qPCR, normalized to *Gapdh.* Graphs show mean + SEM relative to sham-infected WT mice. (**B-C**) The indicated mice were subjected

to OPC. Fungal burdens were assessed at day 5 p.i. (**B**) and *Il22* mRNA was assessed by qPCR normalized to *Gapdh* (**C**). Data is from one experiment. (**D-E)** The indicated mice were subjected to OPC. Fungal burdens were assessed at day 5 p.i. (**D**) and *Il22, Il17a and Il17f* mRNA were assessed by qPCR normalized to *Gapdh* at day 2 p.i. (**E**). Data is pooled from one to two experiments. **(F-H**) WT or *Il22TdTomato* mice were infected with the indicated *C. albicans* strains. Tongue *Il22* mRNA was assessed by qPCR on day 2 p.i. normalized to *Gapdh* (**F**). Data were pooled from 2 independent experiments. Tongue homogenates were prepared 2 days p.i. Cells were gated on the live $CD45^{+}TdTomato^{+}$ population. (**G**) Representative FACS plot is shown above. Summary graphs are shown below. Data are representative of 2 experiments. Each dot represents cells pooled from 2 mice tongues. (**H**) Fungal burdens were assessed at day 5 p.i.. Data was analyzed by T test, and ANOVA. *p<.05, **p<.01, ***p<.001, ****p<.0001

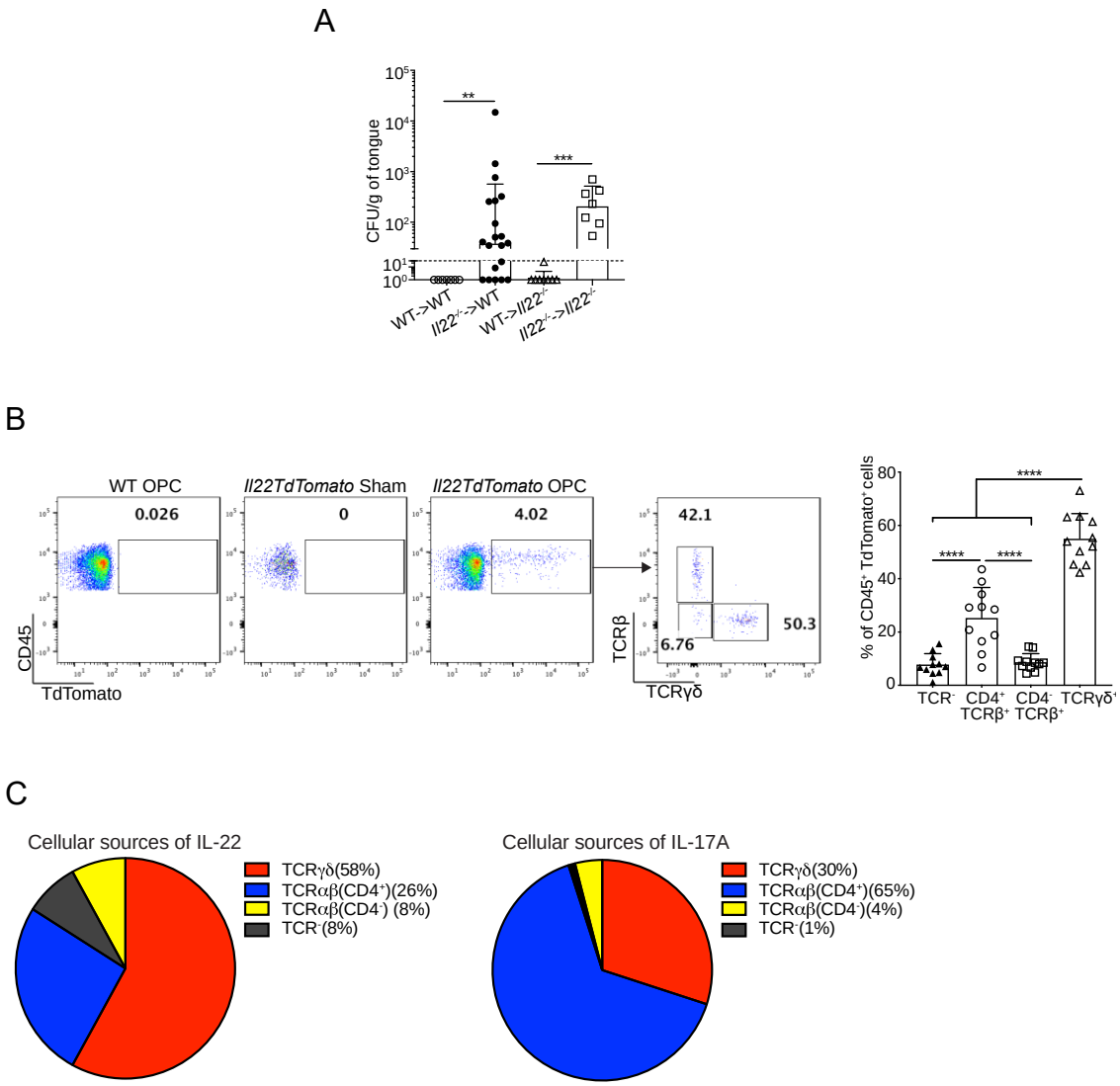

Figure 3.5: Cellular sources of IL-22 during OPC

(A) BM from the indicated donors was transferred into irradiated recipients. After 6-9 weeks, chimeric mice were subjected to OPC and fungal burdens assessed on day 5 p.i. Data were pooled from 2 experiments. (**B)** On day 2 p.i., tongue homogenates from *Il22TdTomato* mice were stained for the TCRβ and TCRγδ and gated on the live $CD45^{+}TdTomato^{+}$ population. *Left*: representative FACS plots. *Right*: Pooled results from 3 independent experiments. Each symbol represents data from 2 pooled tongues. (**C)** Comparisons of the relative percentages of $IL\text{-}22^{+}$ cells (left) or IL-

17^+ cells [data from [37]] isolated from tongues of mice 2 days p.i. and analyzed by flow cytometry. Data were analyzed by ANOVA or Student's t-test, with Mann-Whitney correction for fungal load analysis. **p<.01, ***p<.001, ****p<.0001

3.2.5 Non-hematopoietic cells are the major responders to IL-22 during OPC

IL-22 signals through a heterodimeric receptor complex composed of IL-22RA1 and IL-10R2. While IL-22RA1 is mainly found in non-hematopoietic cells, IL-10R2 is more broadly expressed. IL-22RA1 is also shared with other IL-10 family cytokines beside IL-22 [255]. We sought to compare the susceptibility of *Il22*$^{-/-}$ mice and *Il22ra1*$^{-/-}$ mice to OPC. *Il22ra1*$^{-/-}$ mice showed a very similar, albeit not statistically identical, susceptibility to OPC compared to *Il22*$^{-/-}$ mice (**Fig. 3.6A**). The difference could be indicative of a contribution of other IL-22RA1-dependent cytokines, though this possibility was not pursued. By 14 days p.i., most *Il22ra1*$^{-/-}$ mice had cleared the infection (**Fig. 3.6B)**, which contrasts with *Il17ra*$^{-/-}$ mice that were previously shown to maintain oral fungal loads as long as 17 days p.i. [74, 88]. Contrary to the predominant role of IL-22RA1 in non-hematopoietic cells, some recent studies show the expression of IL-22RA1 in macrophages during tuberculosis and pneumonia infections in the lungs [180, 181]. To identify cell compartments requiring IL-22RA1 in OPC, we created reciprocal BM chimeras with WT and *Il22ra1*$^{-/-}$ mice. Regardless of BM source, WT recipients were resistant to OPC, whereas irradiated *Il22ra1*$^{-/-}$ recipients failed to clear *C. albicans* (**Fig. 3.6C**). Thus, IL-22RA1-mediated signaling in non-hematopoietic cells is required for effective oral antifungal immunity.

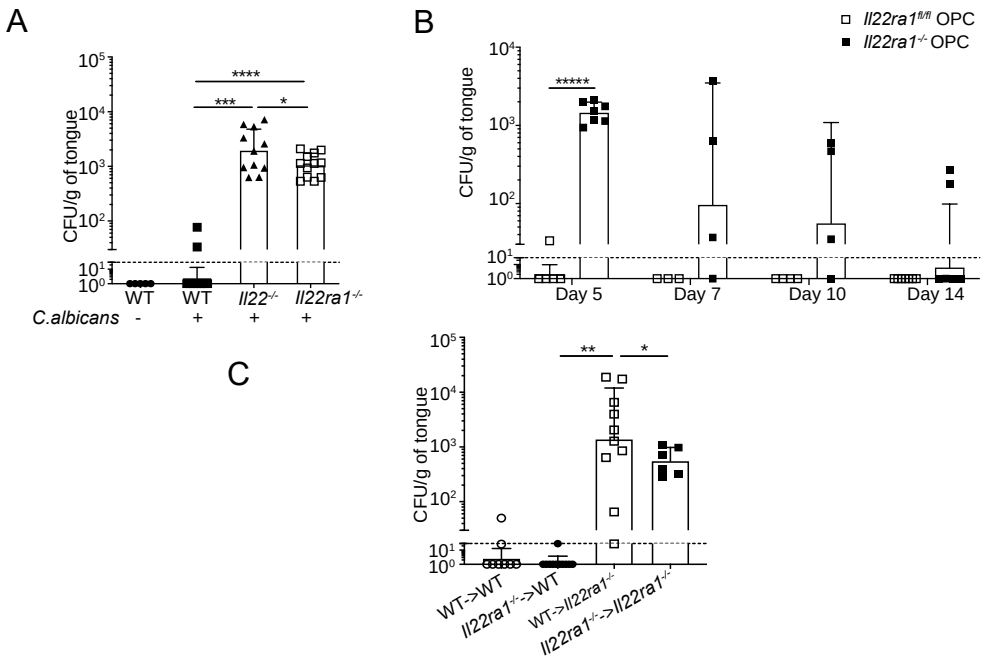

Figure 3.6: IL-22RA1 in non-hematopoietic cells is indispensable for protection against OPC

The indicated mice were subjected to OPC. **(A)** Fungal burdens were assessed on day 5 p.i.. Bars show geometric mean + SD. Data were pooled from 2 experiments. (**B)** *Il22ra1*$^{fl/fl}$ and *Il22ra1*$^{-/-}$ mice were subjected to OPC. Fungal burdens were assessed at days 5, 7, 10 and 14 p.i.. Bars show geometric mean + SD. Data were pooled from 1-2 experiments (**C**) BM from indicated donors was transferred into irradiated recipients. After 6-9 weeks, mice were subjected to OPC and fungal burdens assessed on day 5 p.i.. Data were pooled from 2 experiments and analyzed by ANOVA or Student's t-test, with Mann-Whitney correction. **p<0.01, ***p<0.001, ****p<0.0001

3.2.6 IL-22 signaling in the oral basal epithelium is required for protection against OPC

In view of the observed predominant role of IL-22RA1 in non-hematopoietic cells, we sought to determine more precisely where IL-22RA1 was expressed and functional in the tongue

during OPC. Oral epithelial tissue is characterized by morphologically distinct expression of cytokeratins. Keratin 13 (K13) is expressed in differentiating SEL cells in the post-mitotic, terminally differentiated layer, which overlays the proliferative keratin14 (K14) expressing BEL [80]. Consequently, $K13^+$ epithelial cells make first contact with *C. albicans* during hyphal invasion and are highly subject to fungal-induced tissue damage. Moreover, this SEL layer is then sloughed and swallowed as part of the antimicrobial clearance response [3]. The SEL is replenished by proliferation of the underlying basal $K14^+$ cells which have stem-like properties, but how this is controlled in OPC is unclear. Immunofluorescent (IF) staining indicated that IL-22RA1 was prominent in the $K14^+$ BEL, with some staining in $K13^+$ SEL and papillae (**Fig. 3.7A**). Isotype controls verified Ab specificity (**Fig. 3.7B-C**).

To define the relative contributions of IL-22RA1 in oral epithelial cell subtypes, $Il22ra1^{fl/fl}$ mice were crossed to $K13^{Cre}$ or $K14^{CreERT2}$ mice [88, 256]. Conditional deletion of *Il22ra1* in SEL was efficient, as verified by IF. While the loss of IL-22RA1 staining was evident in the SEL, IL-22RA1 staining was still present in the BEL (**Fig. 3.8A**). $Il22ra1^{K13}$ mice were resistant to OPC (mean fungal load ~19), indicating that IL-22 signaling in SEL is dispensable for fungal control (**Fig. 3.8B)**. Remarkably, these results show that IL-22 signals in a spatially distinct manner from IL-17, because deletion of *Il17ra* in $K13^+$ cells resulted in significant susceptibility to OPC [88]. To delete IL-22RA1 in BEL, $Il22ra1^{K14ERT2}$ mice and controls ($Il22ra1^{fl/fl}$, $Il22ra1^{fl/-K14ERT2}$, $Il22ra1^{K14ERT2}$) were administered tamoxifen for 5 days before they were infected with *C. albicans.* Inducible deletion of IL-22RA1 was efficient in some mice as evidenced by almost complete loss of IL-22RA1 in the BEL (Fig. 3.8C) while others had patchy deletion of IL-22RA1. In contrast to $Il22ra1^{K13}$ mice, loss of *Il22ra1* in $K14^+$ cells led to markedly increased susceptibility to OPC (fungal load ~508), demonstrating that IL-22R signaling is commensurate with its expression

profile in the BEL (**Fig. 3.8D**).

Hepatic IL-22R was previously shown to be needed for pulmonary bacterial immunity [221], so we subjected mice with a liver-specific deletion of IL-22RA1 (*Il22ra1*^{Alb}) to OPC. As shown, they were resistant to OPC (**Fig. 3.8E**). Hence, these data show that IL-22RA1 signaling in the K14^{+}BEL, but not the K13^{+}SEL, is required for oral fungal control, and accordingly that IL-17 and IL-22 function in different epithelial compartments.

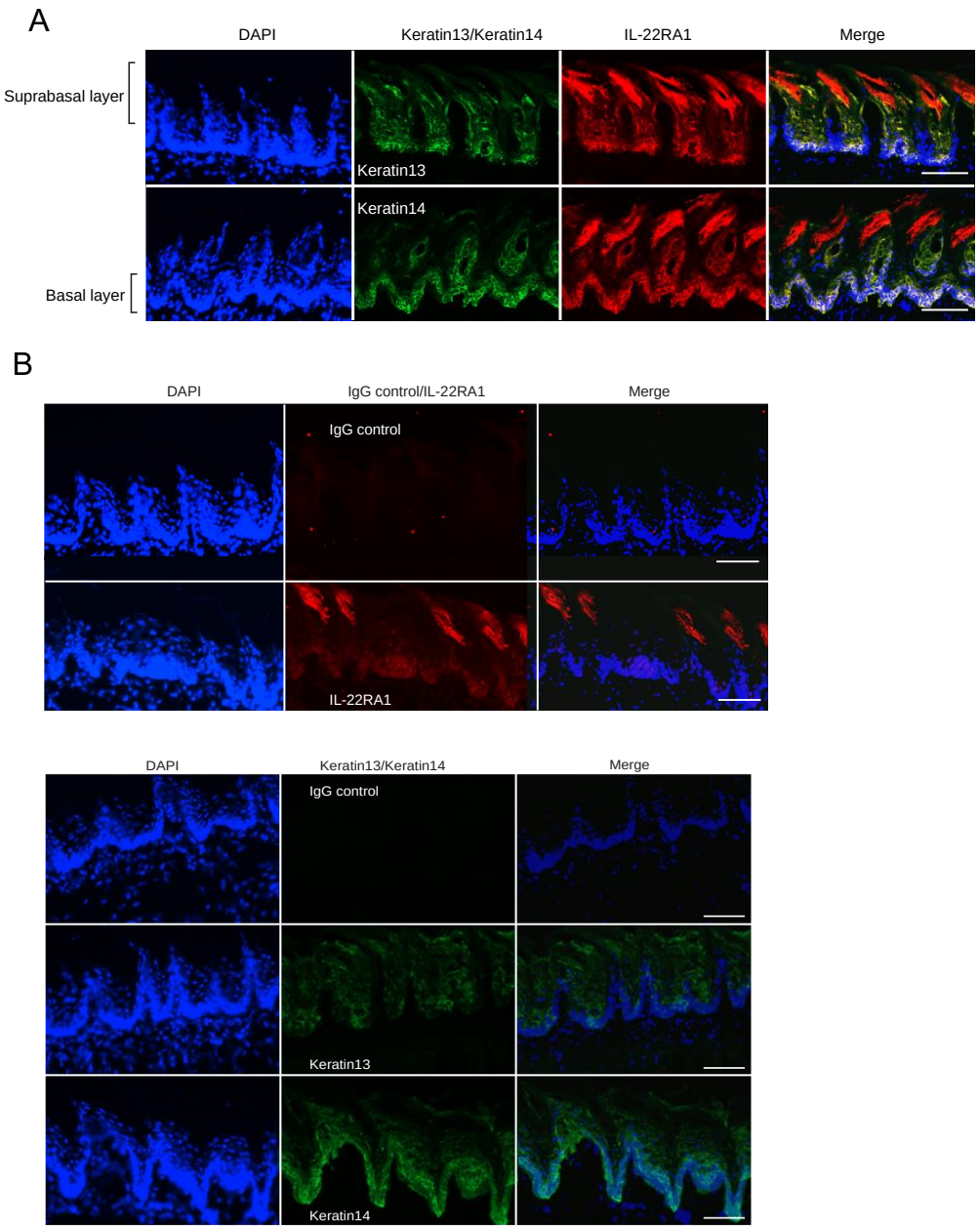

Figure 3.7: IL-22RA1 is expressed in the oral suprabasal and basal epithelial layers

Frozen sections from WT tongues were co-stained with DAPI and Abs against (**A**) K13, K14, IL-22RA1and (**B**) isotype controls. Suprabasal and basal epithelial layers are indicated. Images are representative of a minimum of 3 sections. Size bar = 200 µM

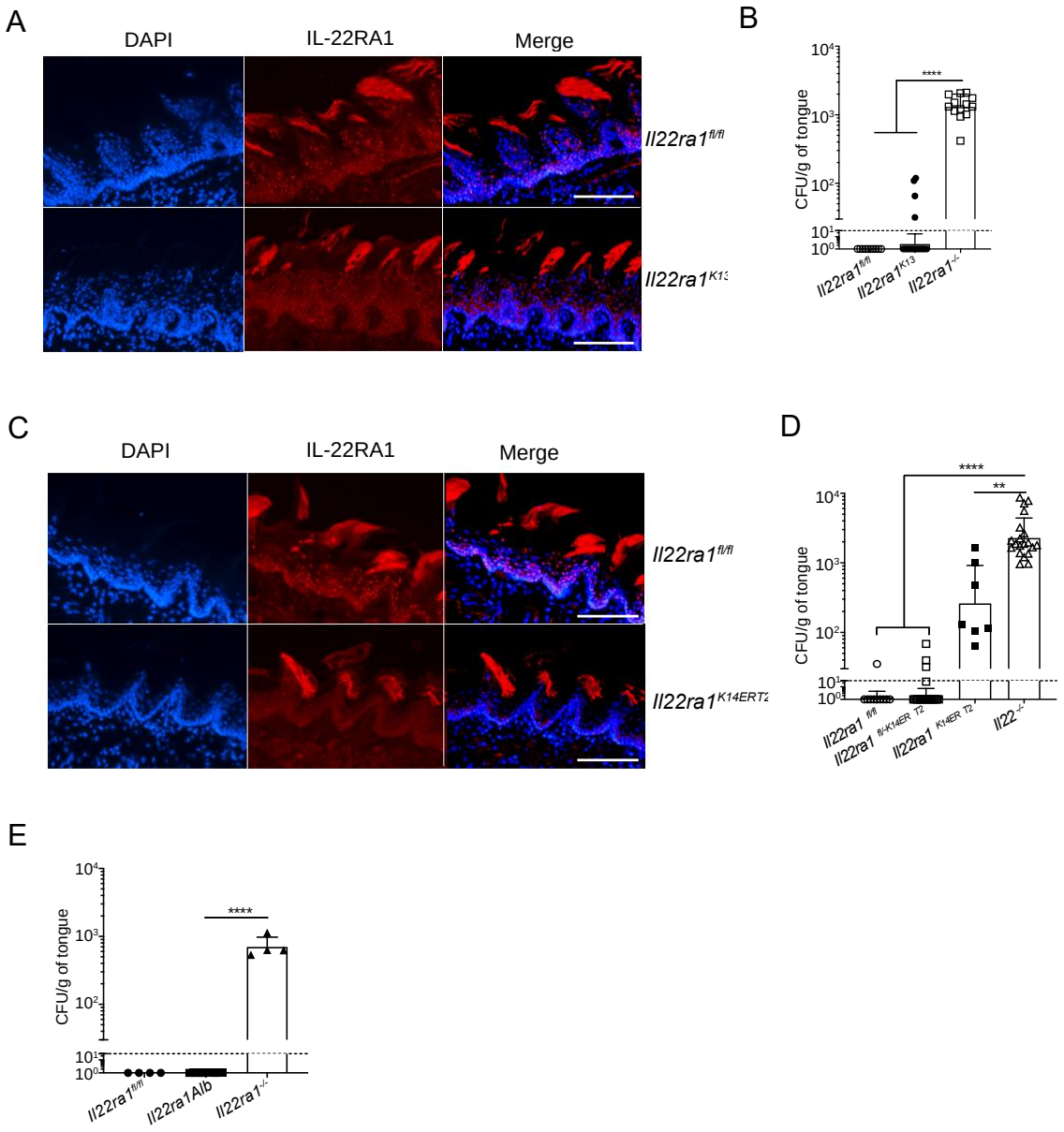

Figure 3.8: IL-22 signaling in the basal epithelial layer is required to control OPC

(**A** and **C)** IF staining of IL-22RA1 in tongue sections from the indicated mice. Size bar = 200 µM. (**B**, **D** and **E**) Fungal burdens were assessed on day 5 p.i. in the indicated mice. In (**D**), all except *Il22*$^{-/-}$ mice were administered tamoxifen for 5 days prior to OPC. Bars show geometric mean + SD. Data were pooled from 3 experiments for (**B and D**) and one experiment for **E**. Data were analyzed by ANOVA with Mann-Whitney correction. **p<.01, ****p<.0001.

3.2.7 IL-22 promotes BEL proliferation and survival during OPC

To determine the mechanisms by which IL-22 promotes immunity during OPC and whether this differs from IL-17-driven responses, we evaluated RNASeq profiles of total tongue mRNA from *C. albicans*-infected *Il22*$^{-/-}$, *Il17ra*$^{-/-}$ and WT mice. As shown, there was differential expression of genes in *Il22*$^{-/-}$, *Il17ra*$^{-/-}$ and WT mice (**Fig 3.9A**). In keeping with the observation that IL-17 and IL-22 act non-redundantly, there were distinct gene sets induced by IL-22 (368 genes) versus IL-17RA (931 genes). There were also many overlapping genes controlled by both cytokines (215 genes) (**Fig. 3.9B**).

GSEA revealed increased expression of mitotic spindle checkpoint genes in WT versus *Il22*$^{-/-}$ mice (**Fig. 3.10A**), normalized enrichment score (NES) 1.4 ($P<0.01$). Specifically, positive cell cycle regulatory genes (*Stat3, Jun, Sphk1*) were reduced and negative regulators of cell cycle (*Cdkn1c, Ddit3, Ets1*) were elevated (**Fig. 3.10B**). Concordantly, *Il22ra1*$^{-/-}$ mice showed decreased IF staining of Ki67 in K14^{+} cells following *C. albicans* infection, indicating that IL-22 is a major driver of BEL proliferation (**Fig. 3.10C**). Consistently, there was a trend to decreased bromodeoxyuridine (BrdU) incorporation in *Il22*$^{-/-}$ K14^{+} BEL cells following oral *C. albicans* infection (**Fig. 3.10D**). We next evaluated cell cycle progression in Epcam^{+} epithelial cells from *Il22*$^{-/-}$ and WT tongues by BrdU and 7-aminoactinomycin D (7-AAD) staining. As shown, there were comparable proportions of cells in the Gap2/mitotic (G2/M) phase, fewer in the synthesis (S) phase and more in the G0/G1 phase (**Fig. 3.11**). GSEA also suggested enrichment of cell death-associated and DNA damage response genes (**Fig. 3.12A, Fig. 3.12B**). Indeed, *Il22*$^{-/-}$ mice had higher frequencies of active caspase 3^{+} and TUNEL^{+} epithelial (Epcam^{+}) cells following infection (**Fig. 3.12 C, D, E**). Hence, IL-22 promotes BEL proliferation during OPC, while limiting accumulation of apoptotic cells.

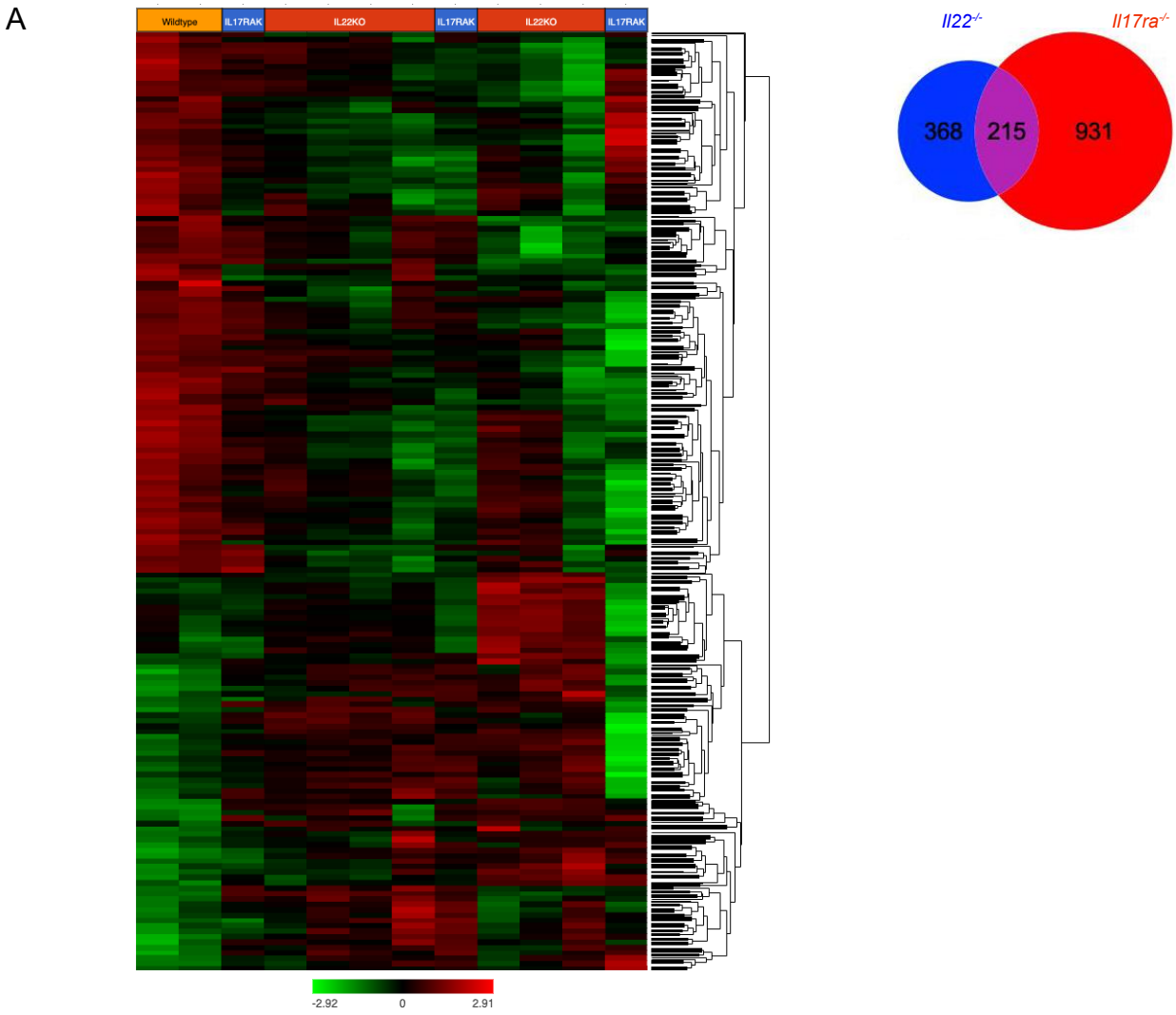

Figure 3.9: Transcriptional profile of *Il22*$^{-/-}$ and *Il17ra*$^{-/-}$ mice during OPC

RNA-Seq was performed on tongue mRNA from WT, *Il22*$^{-/-}$ and *Il17ra*$^{-/-}$ mice, isolated 24 h p.i. (**A**) Heatmap of differentially expressed genes in WT, *Il22*$^{-/-}$ and *Il17ra*$^{-/-}$ mice (parthekflow) **(B)** Venn diagram of differentially regulated or overlapping genes in infected *Il22*$^{-/-}$ and *Il17ra*$^{-/-}$ compared to WT mice. 215 genes were regulated by both IL-22 and IL-17RA, whereas 368 genes were regulated only by IL-22, and 931 genes were regulated only by IL-17RA.

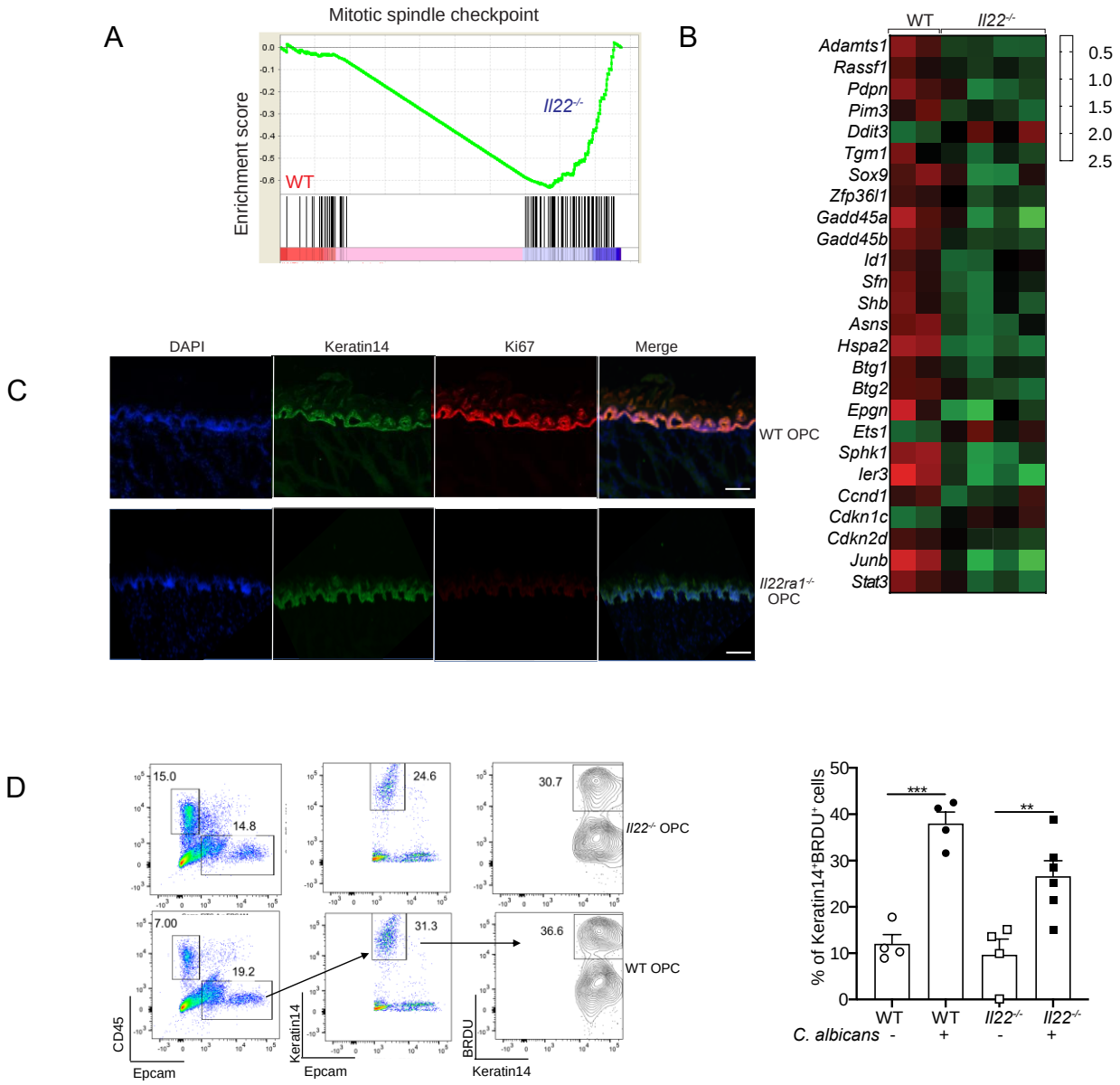

Figure 3.10: IL-22 drives proliferation of basal epithelial cells during OPC

Differentially expressed genes from WT and *Il22*$^{-/-}$ mice were subjected to GSEA. (**A**) GSEA of mitotic spindle checkpoint pathway (**B**) Heatmap of cell cycle pathway genes (Partekflow) in WT or *Il22*$^{-/-}$ mice. (**C**) IF staining of DAPI, Ki67 and K14 in WT and *Il22ra1*$^{-/-}$ mice at 2 days p.i. Data are representative of images from 2 mice per group. Size bar = 100 µM. (D) BrdU was

administered to the indicated mice 24 h p.i., and tongues harvested on day 2 p.i.. Incorporation of bromodeoxyuridine (BrdU) in $CD45^{-}EPCAM^{+}K14^{+}$ cells was assessed by flow cytometry. Left: representative FACS plot, right: summary plot. Data show mean + SEM and were analyzed by ANOVA. **p<0.01, *p<0.001.

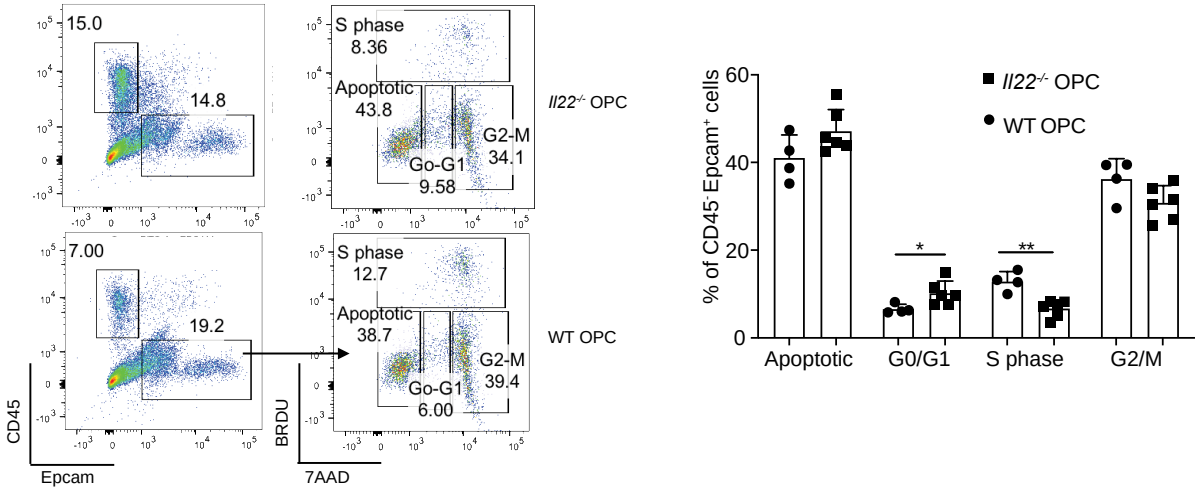

Figure 3.11: IL-22 promotes cell cycle progression in oral epithelial cells during OPC

BrdU was administered 24 h p.i., and tongues harvested on day 2 p.i.. Cell cycle/apoptotic status of $CD45^{-}EPCAM^{+}$ cells was determined by bromodeoxyuridine (BrdU) and 7-aminoactinomycin (7AAD) staining. Left: representative FACS plot, right: summary plot. Data show mean + SEM and were analyzed by Student T test. *p<0.05, **p<0.01.

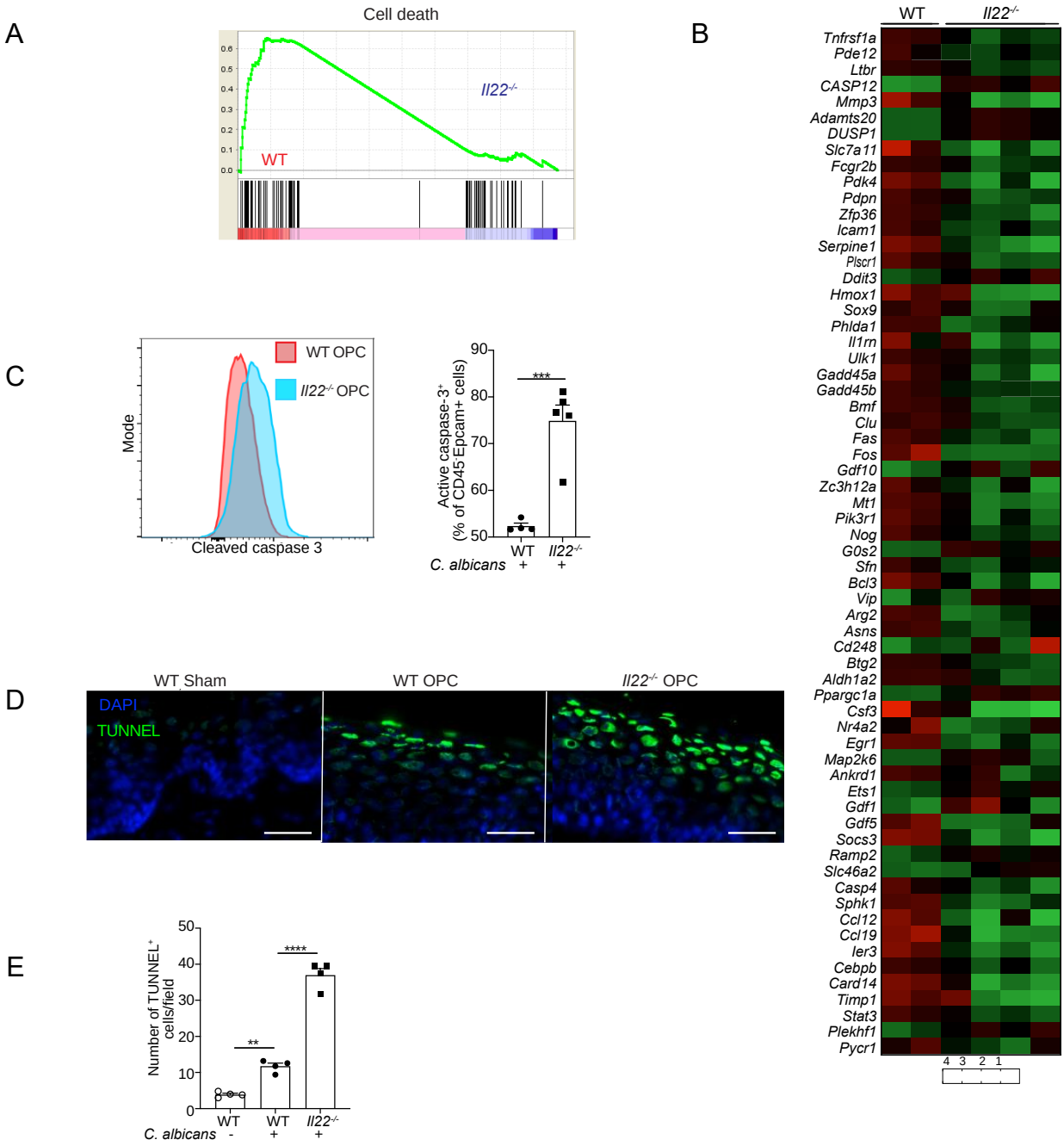

Figure 3.12: IL-22 promotes survival of oral epithelial cells during OPC

Differential gene expression data from WT, *Il22*$^{-/-}$ was subjected to GSEA (**A)** GSEA of cell death pathway genes. Normalized enrichment score is shown on Y-axis (**B**) Heat map of cell death pathway genes (Partekflow) in WT and *Il22*$^{-/-}$ mice in response to OPC. (**C**) Frequency of CD45^{-}

EPCAM$^+$ cells staining positive for active (cleaved) caspase-3 in tongue homogenates at day 2 p.i. measured by flow cytometry. *Left*: Representative FACS histogram. *Right:* Pooled data from 4 independent samples. Data analyzed by student's t-test. (**D**) DAPI and TUNEL staining of tongue sections from the indicated mice at day 2 p.i.. Images are representative of sections from 4 mice per group. Size bar = 200 μM **(E).** Quantification of TUNEL$^+$ cells from panel D. Data analyzed by student T test, ANOVA and post-hoc Tukey's test. **p<0.01, ***p<0.001, ****p<0.0001.

3.2.8 IL-22 restores IL-17R expression and signaling in SEL to sustain antifungal immunity in OPC

In stratified epithelia such as the oral mucosa, proliferating BEL regenerate the post-mitotic SEL [80, 82]. In view of the impaired proliferation of basal epithelial cells observed in *Il22*$^{-/-}$ mice (**Fig**. **3.10C),** I investigated how this defect impacts the oral epithelium. Consistently, genes implicated in tissue repair, keratinization and epithelial differentiation were impaired in *Il22*$^{-/-}$ mice compared to WT mice (**Fig. 3.13A**). Though IL-17RA signaling in K13$^+$ SEL cells is critical for immunity to OPC [88], the factors that restore the SEL after sloughing have not been well defined. In view of the observed role of IL-22 on the BEL, I hypothesized that IL-22 might indirectly impact IL-17 signaling in OPC by restoring the IL-17RA-expressing SEL. Indeed, transcriptomic data and qPCR revealed that there was decreased *Il17ra* mRNA expression in *Il22*$^{-/-}$ tongue (**Fig. 3.13B, C**). IF staining revealed loss of IL-17RA in the SEL of WT mice after *C. albicans* infection, which was even more pronounced in *Il22*$^{-/-}$ mice (**Fig. 3.13D**). Flow cytometry also demonstrated that IL-17RA cell surface expression was reduced in WT and *Il22*$^{-/-}$ CD45$^-$Epcam$^+$ oral epithelial cells following *C. albicans* infection, though the difference between WT and *Il22*$^{-/-}$ mice was modest **(Fig. 3.13E)**. Consistent with reduced IL-17RA in oral epithelium, canonical IL-17 target genes

associated with immunity to *C. albicans* were downregulated in *Il22*$^{-/-}$ mice during OPC, including the essential antimicrobial peptide β-defensin-3 (*Defb3*) (**Fig 3.13F**). Collectively, these data support a model in which IL-22 signaling promotes BEL-intrinsic signals that mediate anti-*Candida* immunity. Accordingly, IL-22 'licenses' IL-17R signaling by renewing the SEL and thereby restoring the capacity of the tissue to respond to IL-17R, which is vital in controlling OPC (**Fig. 3.14**).

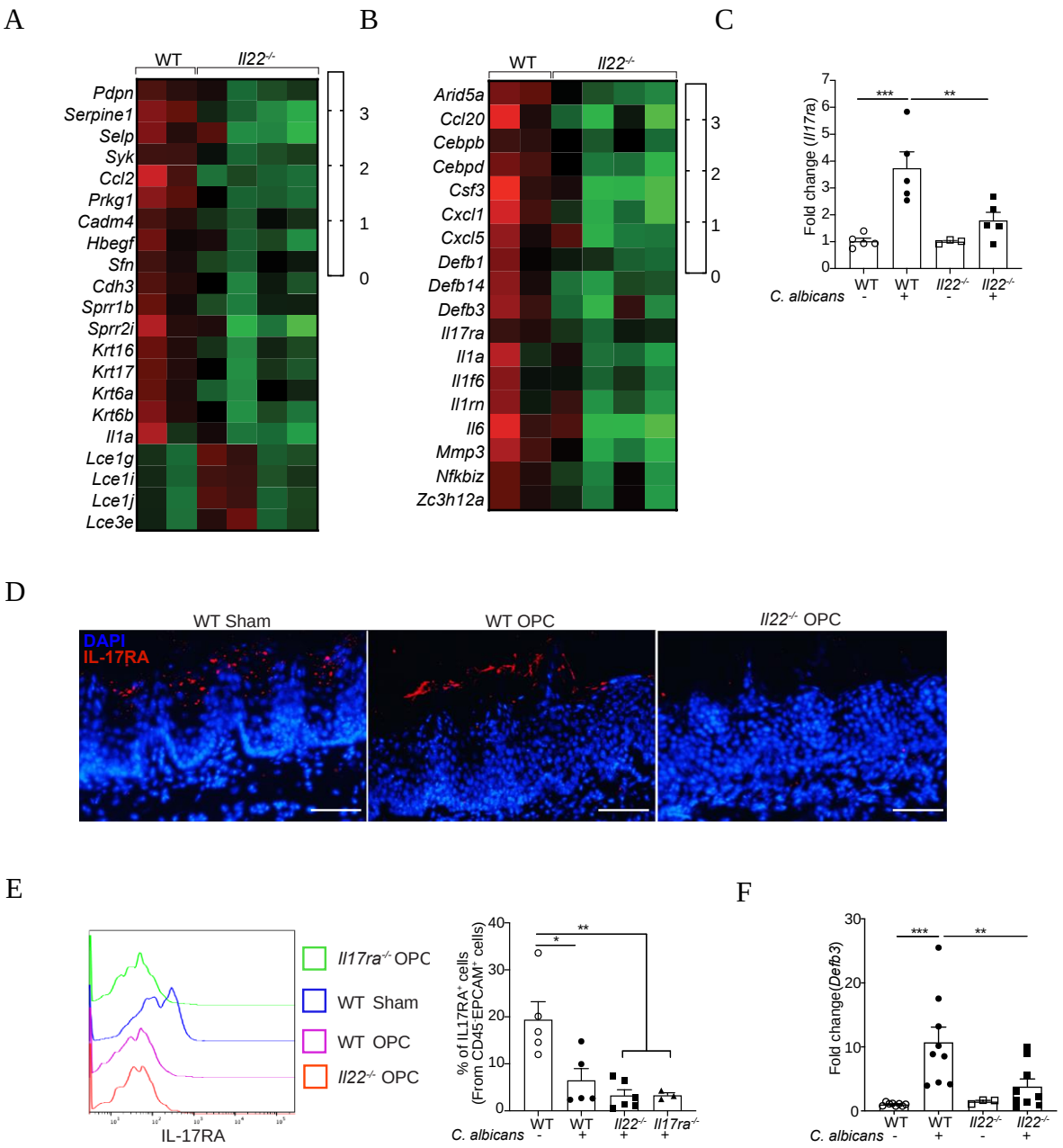

Figure 3.13: IL-22 promotes survival of oral epithelial cells during OPC

(A) Heatmap of genes implicated in tissue repair, wound healing, keratinization and epithelial differentiation. (**B)** Heatmap of IL-17 signature genes in OPC. (**C)** qPCR of *Il17ra* expression normalized to *Gapdh* in tongue tissue from the indicated mice subjected to OPC and analyzed on day 2 p.i.. Data show mean + SEM relative to sham-infected mice. Data analyzed by ANOVA or

student's t-test**. (D)** IF staining of IL-17RA and DAPI in the indicated mice on day 2 p.i. Size bar = 200 µM (**E)** IL-17RA expression in $CD45^{-}Epcam^{+}$ oral epithelial cells in WT or *Il22*$^{-/-}$ mice during OPC. Left: representative FACS histogram. Right: Pooled data from 2 independent experiments. (**F)** Expression of BD3 (*Defb3*) mRNA in tongue from WT or *Il22*$^{-/-}$ mice during OPC, normalized to *Gapdh*. Data analyzed by ANOVA or student's t-test. *p<0.05, **p<0.01, ***p<0.001,

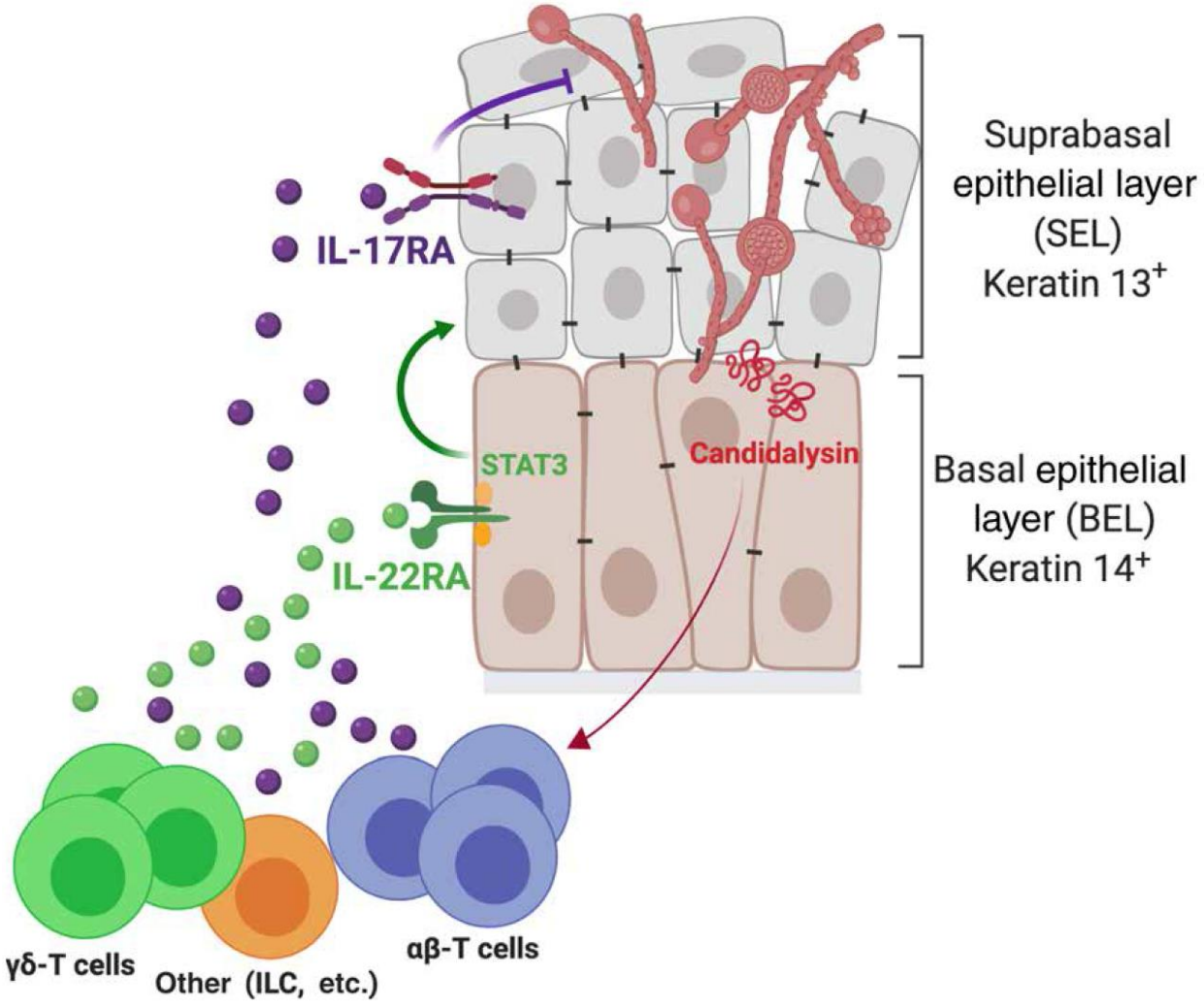

Figure 3.14: Model diagram depicting *C. albicans* encounter with the stratified oral epithelium

Fungal hyphae induce cellular damage and secrete the peptide candidalysin, which facilitates tissue invasion and activates innate IL-17- and IL-22-producing lymphocytes (see Refs [34, 37]. IL-17 was shown previously to act dominantly on $K13^+$ cells of the suprabasal epithelial layer (SEL) [88]. In contrast, IL-22/STAT3 promotes proliferation of the $K14^+$ basal epithelial layer (BEL) that serves to restore the IL-17R-expressing SEL, thus maintaining IL-17-induced antifungal signals such as β-defensins and neutrophil responses that are required to mediate clearance of *C. albicans.* Diagram created on Biorender.com.

3.3 Discussion

Although IL-22 and IL-17 are both produced by Type 17 cells, these cytokines are distinct in structure, receptors, and downstream signaling pathways [202]. Even so, they are usually viewed interchangeably in the context of mucosal immunity. The protective function of IL-22 in oral candidiasis has been recognized for some time, yet its mechanisms of action are incompletely understood. In non-oral manifestations of *C. albicans* infection or colonization (systemic, vaginal, dermal or gastric), IL-22 plays surprisingly different roles, emphasizing that specific cytokine responses even to the same pathogen are influenced by tissue milieu [74, 238, 257-260].

While multiple families of CMC patients have been identified with mutations in IL-17R pathway genes [109, 261, 262], thus far no humans with genetic IL-22-deficiencies have been reported. Nonetheless, it does not necessarily follow that this cytokine is unimportant in humans, only that its contribution to host defense is more modest than that of IL-17. This would be analogous to the role of IL-17F. In mice, loss of IL-17F alone does not cause OPC, yet dual blockade of IL-17F and IL-17A significantly increases susceptibility [241, 247]. In humans, a

family with IL-17F mutations has been described with CMC, suggesting a role in mucosal candidiasis [109]. *AIRE*-deficient humans have circulating neutralizing Abs against IL-17F, IL-22 and IL-17A, thought to help explain the CMC manifestations in these patients [100, 106-108]. In most mammals, though not mice, Th17 cells also express IL-26, a member of the same cytokine family as IL-22, and this cytokine may also be a target of biologic therapy [263]; however, there is no evidence for autoantibodies against IL-26 in *AIRE* deficiency nor are IL-26-deficient humans described [264].

The mechanisms by which IL-17 and IL-22 act in OPC are divergent [202]. Whereas loss of IL-17 impairs neutrophil recruitment [74, 88], IL-22 deficiency led to increased neutrophil tissue infiltration, presumably because *Il17a* is concomitantly elevated. These opposing activities on the neutrophil response may help to restrain excessive inflammation. Interestingly, the capacity of IL-17 to drive oral neutrophil recruitment in OPC is not observed by all who use this OPC model [129, 136], possibly reflecting altered microbiota or other distinctions among animal facilities. Hence, the events controlling oral mucosal immunity are complex and dynamic.

The immunology of the oral cavity is less well understood than other mucosae [3, 234]. In part, this is due to technical challenges associated with isolating cells from oral mucosal sites and the paucity of tools available to interrogate cell types within this tissue [242]. We show here that IL-22 and IL-17 are produced by and function in distinct oral cell subtypes in the setting of OPC. Unlike humans, mice do not harbor *C. albicans* as a commensal organism, and hence acute oral infection with *C. albicans* reflects an innate, not adaptive, immune response. In prior studies, IL-17 was shown to be produced by several innate lymphocyte subsets, including TCR$\alpha\beta^+$ 'natural' Th17 cells (nTh17), which express CD4, have a diverse TCR repertoire, and are activated in a non-antigen-specific manner [37, 86]. IL-17 is also detected to a lesser extent in γδ-T cells and, in some

reports, ILC3 cells [37, 86, 241]. Upon a recall encounter to *C. albicans*, mice generate conventional, antigen-specific Th17 cells that additionally contribute to the IL-17-producing pool, where they augment immunity to *C. albicans* [53, 243, 244]. *C. albicans*-specific Th17 cells are abundant in humans, as *C. albicans* is encountered very early in life. Moreover, *C. albicans*-specific Th17 cells recognize and provide cross-reactive protection against other fungal species, which is likely why maintaining *C. albicans* as a commensal is evolutionarily advantageous [151, 265-267].

We observed that γδ-T cells were the predominant oral source of IL-22 during OPC, followed by nTh17 cells and TCR-negative cell types. These results parallel observations made in skin upon *C. albicans* infection, where γδ-T cells are the major source of Type 17 cytokines. [116]. Since the sources of these cytokines were made using reporter mice which may under-report, defining the relative differences in sources of IL-17 and IL-22 is worth pursuing in more detail [254, 268]. Nonetheless, the induction requirements for IL-22 during OPC are remarkably similar to that of IL-17; namely, IL-23 and the fungal peptide candidalysin are crucial, whereas classical fungal PRRs such as Dectin-1, CARD9 or activators of conventional Th17 cells such as AhR or SAA1/2 are dispensable [37, 53, 252, 253]. The finding that AhR was not essential to induce IL-22 or clear *C. albicans* from the mouth, although initially unexpected, is consistent with observations that AhR facilitates EGFR phosphorylation in OPC, a key step in fungal adhesion, endocytosis and invasion in OECs [23, 24, 36, 58].

IL-17 and IL-22 are typically depicted signaling on the same cell types to mediate mucosal immunity, which is the case in non-stratified epithelia such as gut or lung [159, 202]. Hence, we did not anticipate that IL-17 and IL-22 would act upon spatially distinct cell types in the oral mucosa. To show this, we made use of a mouse that expresses Cre under control of the murine

Krt13 proximal promoter, one of the first tools allowing relatively specific deletion in the oral mucosa [88]. The *K13*^{Cre} mouse deletes conditionally in the SEL of the tongue, buccal mucosa, esophagus and vaginal tract, with no detectable Cre activity in BEL of any tissue examined. Using this system, we found that IL-17RA acts dominantly within K13^{+} cells in the setting of OPC, with fungal burdens only slightly reduced compared to a full *Il17ra*$^{-/-}$ animal [88]. In contrast, deletion of IL-22RA1 in K13^{+} SEL did not impact fungal clearance during OPC. GSEA of RNASeq data predicted a role for IL-22 in tissue proliferation and repair during *C. albicans* infection. Indeed, IL-22/IL-22R signaling was vital for proliferation and survival of the K14^{+}BEL, consequently replenishing the SEL. Thus, IL-22 indirectly permits essential IL-17-driven antifungal events to occur by restoring the post-mitotic superficial layer where IL-17RA is expressed. This spatial stratification of the IL-22R versus the IL-17R enforces the specificity, diversification, and integration of cues that ensure oral epithelial integrity, restrain undue inflammation and promote antifungal immunity.

The oral mucosa is among the most resilient epithelial surfaces [75]. By virtue of their location, superficial K13^{+} OECs are the first to make contact with *C. albicans*. In its non-invasive yeast (commensal) form, *C. albicans* causes no damage to the SEL, which was recently shown to be due to the fact that this form of the fungus does not produce the pore-forming virulence factor candidalysin [34]. Accordingly, there is insufficient expression of IL-1 or other DAMPs that would activate innate lymphocytes to produce IL-17 or IL-22. This creates an environment where benign commensal *C. albicans* colonization is favored. However, in conditions that are conducive to hypha formation and invasion into tissue, a different scenario ensues. As part of the response to fungal invasion, the damaged SEL is sloughed and swallowed, which helps to clear *C. albicans*. The resulting epithelial cell damage also triggers production of IL-1 and IL-36, which promote IL-

17 and IL-23 expression, respectively [37, 117, 135, 136]. IL-17R signaling in the SEL upregulates chemokines that recruit neutrophils as well as β-defensins that exert direct antifungal activity [74, 88, 131, 269]. Hyphal invasion thus establishes an inflammatory milieu that is initiated by candidalysin-induced SEL damage, potentiated by IL-17- and candidalysin-induced effectors such as IL-1 and IL-36, and ultimately resolved upon clearance of the pathogen [37, 135].

IL-22 acts in many epithelial surfaces. Events in the oral epithelia are reminiscent of skin, where $K14^+$ stem cells resupply superficial epithelial cells to maintain barrier integrity [81, 82]. The skin also possesses 'memory' properties that accelerate tissue repair after future insults, though whether this occurs in the mouth is unknown [270]. IL-$22RA1^+$ epithelial cells in colonic epithelium maintain genome integrity and limit apoptotic cell accumulation during genotoxic stress [216]. Similarly, IL-22 signaling in the BEL during OPC promotes replacement of damaged epithelial cells, prevents accumulation of inflammatory apoptotic cells, preserves genome integrity and, as shown here, helps maintain IL-17-driven antifungal activities.

IL-22 maintains the intestinal epithelial barrier during intestinal colonization of *C. albicans* [196, 238]. Unlike the mouth, IL-17 is not protective in gut but rather promotes a tissue-destructive inflammatory cycle in response to *C. albicans* colonization [238]. In fact, IL-17 signaling in gut is generally more reparative than inflammatory [271-275], which is thought to explain why anti-IL-17 biologic therapies failed in trials of Crohn's disease [276]. Anti-IL-17 biologics are associated with low but statistically significant rates of OPC, though it is rare for patients to stop therapy for this reason [277]. Anti-IL-22 antibodies are under evaluation for skin pathologies such as atopic dermatitis [278], but rates of *C. albicans* infections have not been reported. Our data predict that combinations of anti-IL-17 and anti-IL-22 could result in more severe mucosal candidiasis infections than either therapy alone.

In summary, these results reveal a deeper understanding of the antimicrobial defense functions of oral epithelial cells and a complex interplay between distinct cytokines of the Type17 axis. It is clear that cells within the oral epithelium are not simply physical barriers but are topographically structured sentinels that work in concert to dictate the outcome of oral *C. albicans* colonization, the most common fungal infection of humans [99, 279].

4.0 Oral Epithelial STAT3 Signaling is Required for Oral Antifungal Immunity

4.1 Background

Recent studies are changing the view of the role of epithelial cells in dictating the outcome of immune responses at mucosal sites. Beside triggering antimicrobial peptide responses, epithelial cells actively serve as "crosstalk platforms" to relay cues from infecting pathogens and other pathological insults to the immune system. In the gut, intestinal epithelial cell interactions with the microbiome modulate local and systemic immune responses. Segmented filamentous bacteria (SFB) has emerged as one of the key commensals driving intestinal Th17 responses. During SFB colonization, IL-22/STAT3 dependent activation of serum amyloids (SAA1/SAA2) promotes Th17 differentiation and IL-17 production [252]. Oral epithelial damage induced by the *C. albicans* toxin candidalysin triggers the expansion of innate-acting CD4 T cells through IL-1 receptor mediated signaling [37]. Thus, the cytokine milieu in the tissue microenvironment shapes the subsequent immunological responses, and the mucosa is central to instigating these responses.

Cytokines including IL-1β, IL-36, IL-23 and IL-22 released in response to *Candida* infection are all capable of activating STAT3 [280-284]. Indeed, many studies show that various cytokine activities converge on STAT3 and NF-κB activation [285-287]. Importantly, the role of epithelial/stromal STAT3 in coordinating the innate and adaptive immune response to chronic inflammation and cancer have been demonstrated [288, 289]. However, the role of epithelial STAT3 in oral antifungal immunity is not well understood.

Evidence for a key role of STAT3 in antifungal immunity came from Job's syndrome/ autosomal-dominant hyper IgE syndrome (AD-HIES) patients who suffer from recurrent oral and

chronic mucocutaneous candidiasis (CMC) due to decreased Th17 generation. These patients harbor a dominant negative STAT3 mutation which accounts for their decreased numbers of IL-17- and IL-22-producing Th17 cells [290, 291]. The decrease in Th17 cells is thought to be due to their hypo-responsiveness to Th17-inducing cytokines, namely IL-6, IL-23 and IL-21 [101, 102, 281, 292-294]. Analogous to the STAT3 dominant negative mutations, patients with a gain of function mutation in STAT1 are susceptible to CMCD. The disease in these patients is attributed to increased STAT1-mediated signaling and a concomitant repression of STAT3 dependent differentiation of Th17 cells [104].

Although these initial studies pointed to the role of STAT3 in Th17 differentiation as the primary cause of increased CMCD in these inherited disorders, STAT3 is a pleiotropic transcription factor with important roles in other cell types. Loss of STAT3 in macrophages promotes spontaneous colitis due to impaired anti-inflammatory response [295-297]. Similarly, deletion of STAT3 in the intestinal epithelium delays wound healing during colitis partly by regulating bacterial colonization through antimicrobial peptides and epithelial cell survival [298]. In contrast, STAT3 in CD4 T cells mediates disease severity in Th17-driven autoimmune conditions including IBD and EAE, highlighting the detrimental role of STAT3 in these contexts [299]. Consequently, the observed role of STAT3 is dependent on the disease context and the types of cells involved. In line with this, studies have demonstrated varied roles for epithelial STAT3. Aside from the canonical nuclear transcriptional roles of STAT3 in driving survival, proliferation, and maintaining stem cell like properties, non-transcriptional and metabolic roles of STAT3 in cellular homeostasis is beginning to emerge [184, 289].

In this study, I investigated the role of oral epithelial STAT3 in oral antifungal immunity. Bioinformatic analysis of RNASeq data revealed that while STAT3 target genes/pathways were

down-modulated in *Il22*$^{-/-}$ mice, those genes/pathways were unexpectedly enriched in *Il17ra*$^{-/-}$ mice during OPC. In vivo, we demonstrated that STAT3 activation is impaired in *Il22*$^{-/-}$ mice but elevated in *Il17ra*$^{-/-}$ mice during OPC. We observed that phosphorylation of STAT3 was impaired in the BEL in *Il22*$^{-/-}$ and *Il22ra1*$^{-/-}$ mice. Consistent with this observation, mice with SEL specific loss of STAT3 were resistant to OPC, whereas deletion of STAT3 in the BEL resulted in increased susceptibility to OPC. These findings demonstrate a critical contribution of BEL STAT3 in oral antifungal immunity and open up new directions to critically access the likely transcriptional and non-transcriptional roles of STAT3 in oral antifungal immunity.

4.2 Results

4.2.1 STAT3 integrates IL-22 and IL-17RA transcriptional networks during OPC

Previous transcriptomic data has shown that STAT3 activity is upregulated in the tongue in response to OPC in WT mice [74]. To determine how IL-22 and IL-17RA signaling impact the activation of STAT3, we subjected our RNASeq data (**Fig. 3.9**) to GSEA. Consistent with the role of IL-22 in STAT3 activation [228], GSEA revealed decreased enrichment in *Il6/STAT3* gene sets (NES 1.87 p= 0.00) in *Il22*$^{-/-}$ mice compared to WT mice (**Fig. 4.1A**). Unexpectedly, IL-6/STAT3 gene sets were enriched in *Il17ra*$^{-/-}$ compared to WT mice (NES 1.4, P<0.05) (**Fig. 4.1B**). Consistently, Ingenuity Pathway analysis identified STAT3 as a central upstream regulator that integrates the IL-22 and IL-17RA transcriptional networks (**Fig. 4.1C**). Within this network, STAT3 was connected to proliferation and apoptosis genes and transcription factors that regulate inflammation such as NF-κB/IκBζ (*Nfkbiz*) and MAPK/AP-1 (**Fig. 4.1C**). In line with these

bioinformatic predictions, IL-22 induced STAT3 phosphorylation and IκBζ expression in TR146 cells, a human oral epithelial cell line that has been used to understand epithelial responses to *C. albicans* [34, 37, 84] (**Fig. 4.2 A, B**). Together, these observations show the synergy between IL-22 and IL-17 signaling and point to the possible role of STAT3 as a key modulator of these responses during OPC.

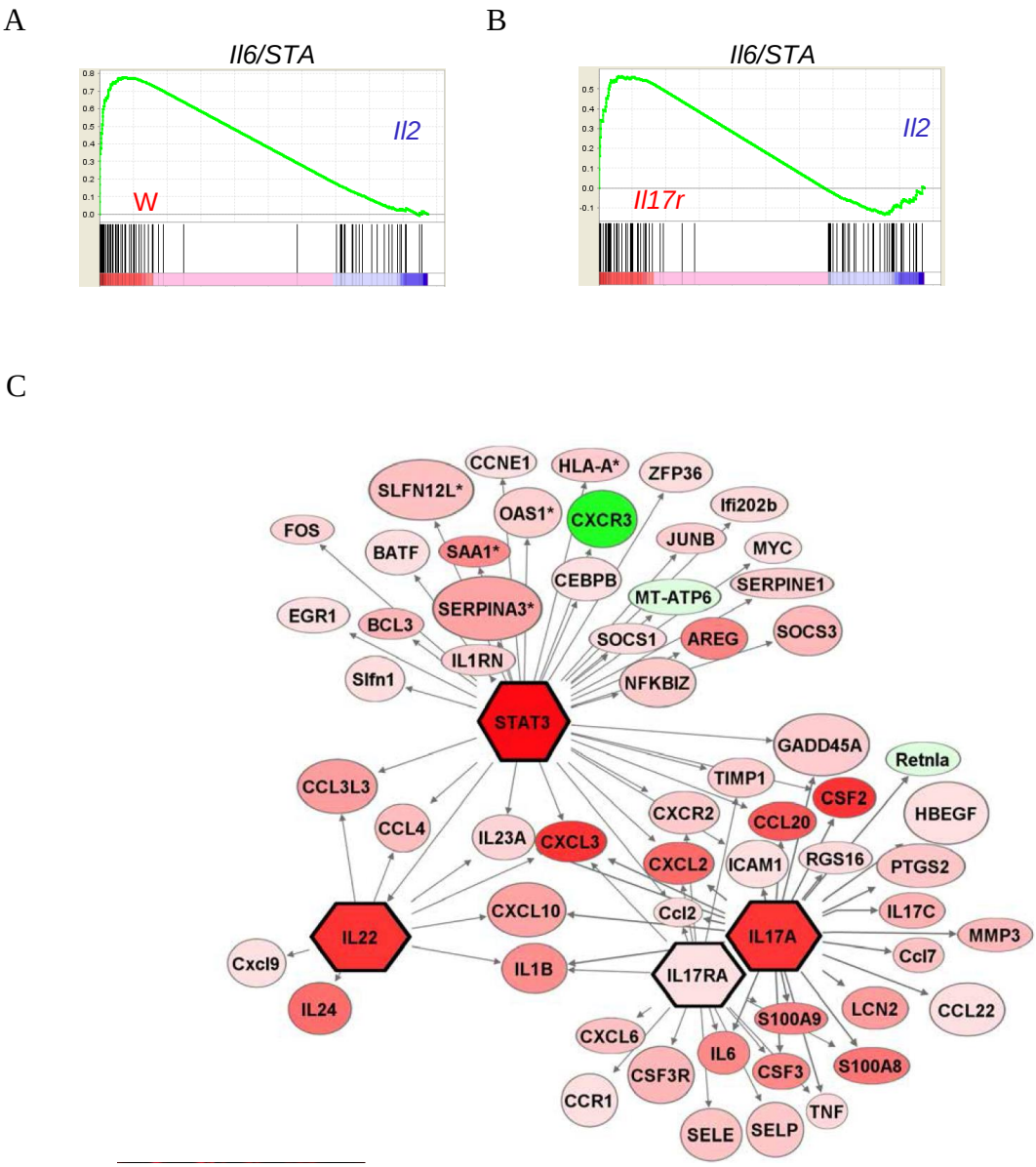

Figure 4.1: STAT3 is a central hub linking IL-22 and IL-17RA signaling during OPC

RNA-Seq was performed on whole tongue mRNA from WT, *Il22*$^{-/-}$ and *Il17ra*$^{-/-}$ mice subjected to OPC and harvested on day 1 p.i.. (**3.9A, B**). GSEA of predicted IL-6/STAT3 gene sets in (**A**) WT and *Il22*$^{-/-}$ mice and (**B**) *Il17ra*$^{-/-}$ and *Il22*$^{-/-}$ mice from ((**3.7A, B**). (**C)**. Ingenuity Pathway Analysis of RNA-Seq data from panel A, indicating that STAT3 is an upstream regulator integrating *Il22* and *Il17ra*- driven transcriptional networks.

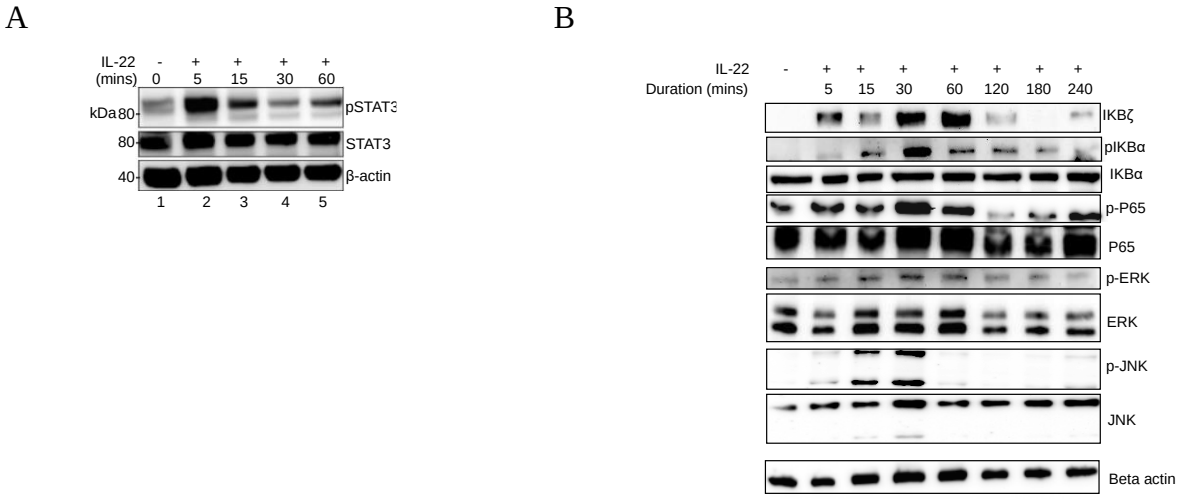

Figure 4.2: IL-22 activates STAT3, NF-κB and MAPK signaling in TR146 OECs

(**A, B**) TR146 OEC were stimulated with or without IL-22 (100ng/ml) for the indicated time points. Lysates were immunoblotted for the indicated proteins. Data are representative of two experiments.

4.2.2 IL-22/IL-22RA signaling activates STAT3 in the BEL during OPC

In view of our bioinformatic and *in vitro* data on STAT3, I sought to assess the state of STAT3 activation during OPC. While STAT3 phosphorylation was impaired in *Il22*$^{-/-}$ and *Il22ra1*$^{-/-}$ mice (**Fig. 4.3A, C)**, STAT3 phosphorylation was increased in *Il17ra*$^{-/-}$ mice, possibly due to elevated *Il22* expression in these mice under these circumstances (**Fig 4.3A, B**). Interestingly,

STAT3 activation was most prominent in the BEL. These results show that, even though numerous stimuli have potential to activate STAT3, the dominant STAT3 response during OPC is mediated by IL-22RA1-dependent cytokines. Moreover, the localization of active STAT3 in the BEL but not SEL demonstrates the compartmentalization of responses in the oral mucosa to *C. albicans* infection. This finding also supports the idea that there is likely to be defective IL-22-driven signaling as part of the pathogenesis of OPC that is associated with *STAT3* mutations (e.g., Job's syndrome). K14 staining intensity by IF was reduced in *Il22ra1*$^{-/-}$ mice compared to *Il22ra1*$^{fl/fl}$ mice (**Fig 4.3C**). This was commensurate with RNASeq data that showed reduced *Krt14* mRNA in *Il22*$^{-/-}$ mice (**Fig. 3.9A, B**), a phenomenon also observed in skin in response to *C. albicans* infections [300].

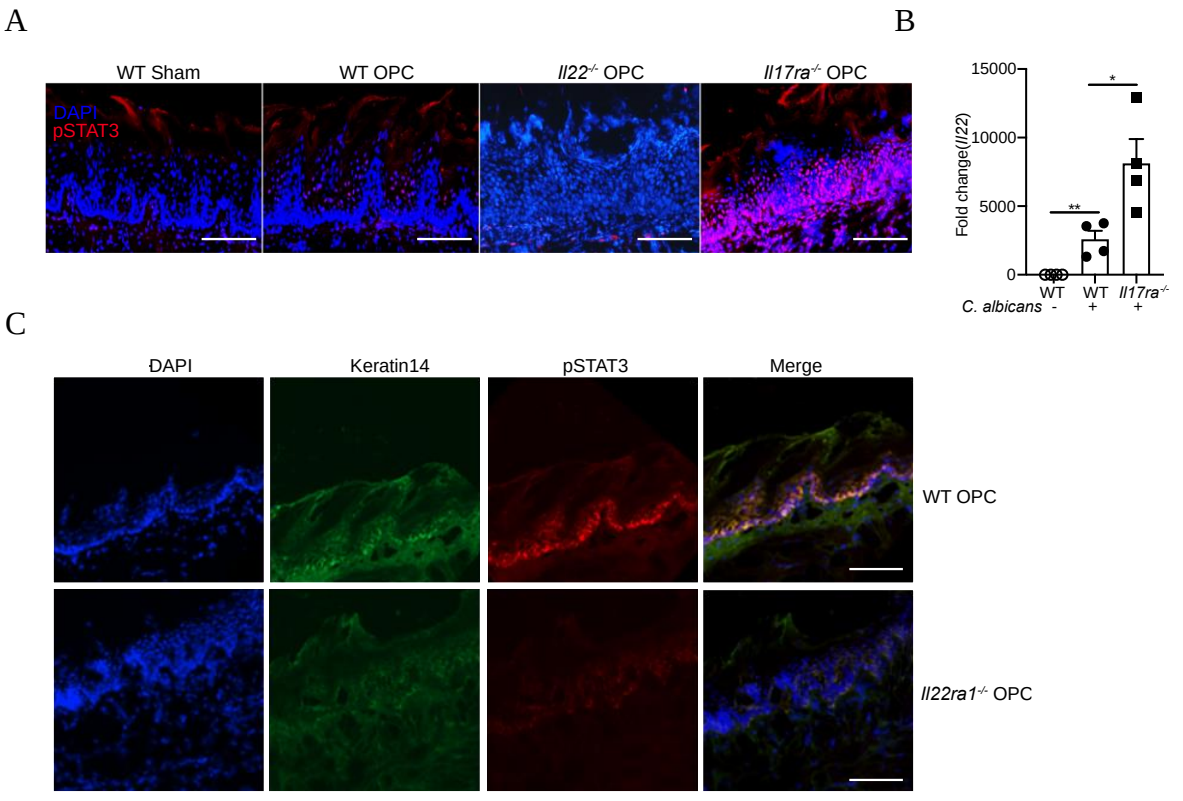

Figure 4.3: STAT3 activation is impaired in BEL of *Il22ra*$^{-/-}$ mice during OPC

(A) IF staining of frozen tongue sections with DAPI and anti-pSTAT3 (Tyr705) in WT, *Il22*$^{-/-}$ and *Il17ra*$^{-/-}$ mice harvested 2 days p.i. . Size bar = 200 μM. (**B)** qPCR of *Il22* induction in tongue harvested from WT or *Il17ra*$^{-/-}$ mice at day 2 p.i. normalized to *Gapdh.* (**C)** IF staining of DAPI, pSTAT3 (Tyr705) and K14 in WT or *Il22ra1*$^{-/-}$ mice at 2 days p.i.. Size bar = 200 μM.

4.2.3 Oral BEL STAT3 is necessary for protection against OPC

To assess the functional relevance of oral epithelial STAT3 in oral antifungal immunity, we generated mice with specific loss of STAT3 in the SEL and BEL using K13^{Cre} or K14^{ERT2Cre} mice respectively. While acute deletion of STAT3 in the BEL rendered mice susceptible to OPC (**Fig. 4.4A, B**), mice with germline STAT3 deletion in the SEL were resistant to OPC (**Fig. 4.5**). Together, these data demonstrate a central role and compartmentalization of STAT3 activation in the oral epithelium to sustain antifungal immunity.

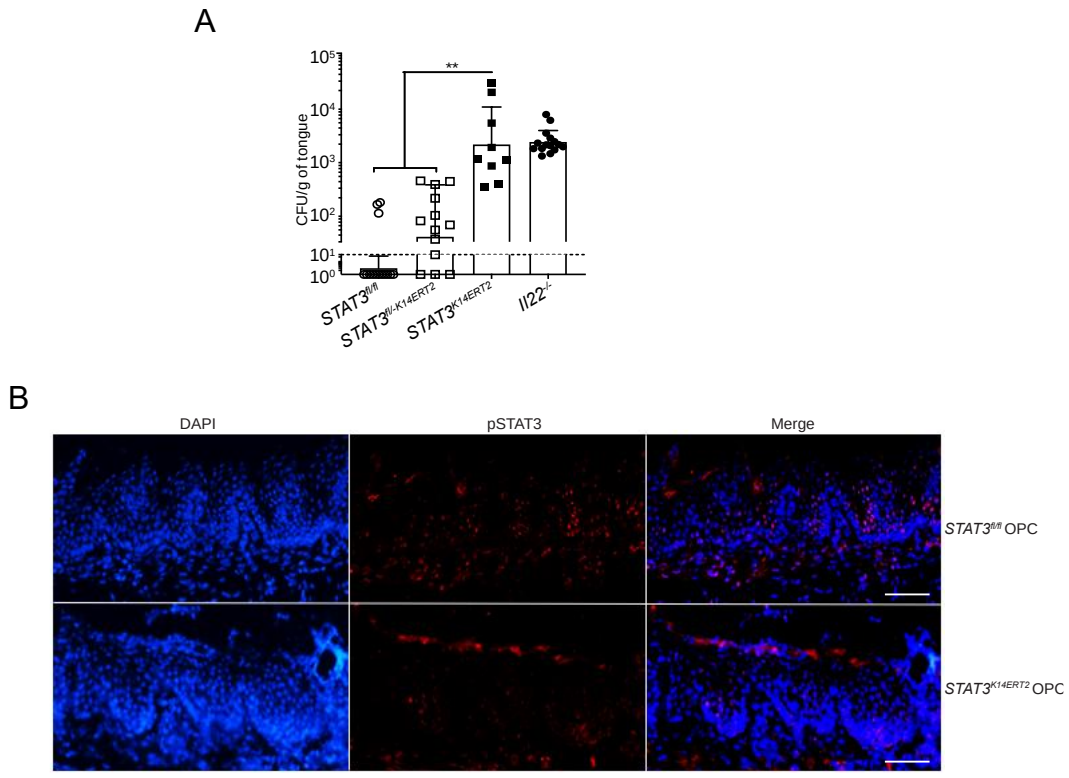

Figure 4.4: STAT3 in oral BEL is indispensable for protection against OPC

(**A**) All mice except *Il22*$^{-/-}$ were administered tamoxifen for 5 days, subjected to OPC and fungal burden assessed on day 5 p.i.. Bars show geometric mean + SD. Data was pooled from 3 experiments. Data analyzed by ANOVA with Mann-Whitney correction. **p<0.01 (B) IF staining of DAPI, and pSTAT3 in the indicated mice at day 2 p.i. Size bar = 200 µM.

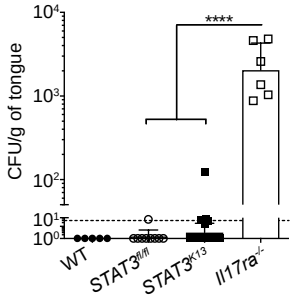

Figure 4.5: Oral SEL STAT3 is dispensable for protection against OPC

The indicated mice were subjected to OPC and fungal burden was assessed on day 5 p.i.. Bars show geometric mean + SD. Data was pooled from 3 independent experiments and analyzed by ANOVA with Mann-Whitney correction. ****$p<0.0001$.

4.3 Discussion

Animal experimental models and 'experiments of nature' in humans that cause primary immunodeficiency syndromes have led to the identification of Th17/IL-17RA axis as a critical driver of antifungal immunity [74, 88, 100, 232, 301]. Impaired Th17 differentiation due to dominant negative mutations in STAT3 as observed in HIES patients is thought to underlie their increased susceptibility to oral candidiasis [101, 102]. However, STAT3 has ubiquitous functions in numerous cell types in different disease settings [295-299]. The oral epithelium is a major orchestrator of antifungal immunity [37, 88]. Nonetheless, the role of STAT3 in oral thrush remains underexplored. In this study, I sought to evaluate the role of oral epithelial STAT3 in OPC.

Multiple cytokines and signaling pathways are known to activate STAT3 [302, 303]. Our GSEA data suggested that STAT3 is an integrating hub between IL-22 and IL-17 signaling networks (**Fig. 4.1C**). This finding was unexpected since IL-17RA is not known to directly activate STAT3. The pathways inferred from bioinformatics data (proliferation, cell cycle, and apoptosis) are commensurate with the known roles of IL-22 and STAT3 in epithelial proliferation and repair [255]. Surprisingly, IL-22/STAT3 genesets (**Fig. 4.1A, B**) and phosphorylation of STAT3 were enriched in the *Il17ra*$^{-/-}$ mice (**Fig. 4.3A**), even though expression of other STAT3-activating cytokines (e.g., IL-6 and G-CSF) were impaired in these mice. This could be due to elevated *Il22*

expression in *Il17ra*$^{-/-}$ mice (**Fig. 4.3B**), but likely reflects overall perturbation of the multiple immune response networks inferred from GSEA that may involve STAT3.

Susceptibility to CMC in Job's syndrome patients with *STAT3* mutations is usually attributed to impaired STAT3-dependent Th17 differentiation [100-102]. However, in acute OPC in mice, STAT3 in CD4^{+} T cells is not required to control fungal loads [86]. Moreover *CD4*$^{-/-}$ and *CD8*$^{-/-}$ mice are resistant to OPC [243]. Unlike humans, mice are naïve to *C. albicans* and are unlikely to have pre-existing *C. albicans* specific Th17 cells. It is therefore likely that STAT3 is not required in Type 17 cells in the acute response to OPC. Thus, STAT3 likely modulates antifungal immunity beyond the hematopoietic compartment. Indeed, our data demonstrate for the first time that STAT3 signaling in the oral BEL is required for protection against OPC (**Fig. 4.3A, 4.4A-C, 4.5**). Supporting this, our group previously showed that HIES patients exhibit impairments in salivary antifungal immunity, with reduced levels of salivary AMPs including β-defensins and histatins [134]. STAT3 deficiency in lacrimal epithelial cells results in decreased $I_KB\zeta$ expression, enhanced apoptosis and a Sjögren's syndrome-like phenotype [304]. Interestingly, $I_KB\zeta$ is key to NF-κB mediated signaling downstream of IL-17RA [126]. STAT3 also regulates metabolism in various settings to meet energy needs for cell proliferation, and STAT3-regulated functions in mitochondria is becoming increasingly appreciated [305-307].

Our findings point to previously unappreciated roles of oral epithelial STAT3 in oral antifungal immunity. While it is conceivable that the IL-22/STAT3 axis coordinates antimicrobial immunity during OPC, it is also likely that oral epithelial STAT3 signaling may exert broader effects in oral antifungal immunity. Our findings therefore open up new questions regarding the role of BEL-specific STAT3 in vital oral antifungal events.

5.0 Unexpected Role of IL-22 in Secondary OPC

5.1 Background

The available human data and data from experimental animals have demonstrated a profound role for Th17/IL-17 signaling in oral antifungal immunity [74, 86, 87, 243, 264]. Although IL-17A is the eponymous Th17 cytokine, Th17 cells also produce IL-22, an IL-10 family cytokine, and is functionally relevant in acute OPC [308]. Unlike mice, humans exert adaptive responses to *C. albicans* due to early exposure in life and *C. albicans* colonizes mucosal surfaces as a commensal fungus in healthy subjects. Importantly, the functions of IL-17 and IL-22 are determined partly by the surrounding milieu. For example, as mentioned earlier, in bleomycin induced lung injury model, IL-22 is only protective in the absence of IL-17 [211]. The local tongue cytokine milieu during innate *C. albicans* response and the spectrum of responding cell types may be different from those induced in the tongue and surrounding draining lymph nodes in recall adaptive settings. In experimental autoimmune encephalitis (EAE), an autoimmune condition mediated by conventional Th17 cells, IL-17 signaling promotes proliferation and survival of lymph node FRCs [309]. While there is very little IL-17 expression or expansion of Th17 cells in the cervical lymph node during innate response to OPC, IL-17 responses are observed during recall *C. albicans* infection [37, 243].

In contrast to humans, mice do not have *C. albicans* as a commensal and therefore lack pre-existing *C. albicans* reactive Th17 cells. Instead, mice mount a robust antigen independent Type 17 response during their initial encounter with *C. albicans* which is complemented by conventional Th17 responses during a second encounter [37, 74, 86, 243]. On the other hand,

humans are exposed to *C. albicans* early in life and generate potent *C. albicans*-specific Th17 cells. Consistent with the antigen-independent activation of innate type 17 cells, *Card9*$^{-/-}$ mice are resistant to acute OPC but are sensitive to recall *C. albicans* infection. This sensitivity is attributed to the failure to generate conventional Th17 responses in *C. albicans* recall infection in *Card9*$^{-/-}$ mice [53]. In addition to IL-17A and IL-17F expression, Th17 cells also produce IL-22. Despite the differences observed in *Card9*$^{-/-}$ mice, the independent roles of IL-17A and IL-22 in recall *C. albicans* infections have not been assessed.

During adaptive immune responses, the surrounding draining lymph nodes are major sites of antigen dependent immune activation, and T cells subsequently egress to the local environment to exert effector functions. The lymph node stromal environment actively dictates lymphoid effector functions [310-313]. In lymphocytic choriomeningitis virus (LCMV) infection, IL-22 deficiency causes thymic and splenic hypertrophy and promotes T cell effector functions in lymphoid organs [217]. IL-22 has also been shown to be important in secondary and tertiary lymphoid organ (SLO and TLO) architecture. In this adenovirus cannulation model, IL-22 is thought to act on TLO stroma to produce CXCL-13 and CXCL-12, which trigger B-cell clustering, lymphoid aggregation and autoantibody production [314]. Similarly, IL-22 promotes thymic epithelial cell regeneration in response to thymic injury, a function that is important for restoring immune competence after physical or pathological insults [140]. IL-22 has also been shown to modulate MHC Class II expression and IFN-γ responses during skin *Staphylococcus epidermidis* colonization [218]. Hence the outcomes of IL-22 deficiency may not be limited to the stromal compartment alone but can also direct lymphocyte functions in a context dependent manner.

These reports indicate that although most immune cells do not express IL-22RA1, IL-22 can have essential indirect immune modulatory functions on lymphocytes. Such activities are

likely to be prominent in settings of chronic inflammation and antigen exposure that promote TLO formation and adaptive responses. I therefore sought to determine the role of IL-22 in the adaptive response using our previously developed oral *C. albicans* recall infection model. In this system, mice are sublingually inoculated with *C. albicans*, rested for 6-8 weeks in order to permit development of adaptive responses, and then re-infected to assess the nature of the immune recall response. As shown in this chapter, I found that IL-22 is induced in secondary OPC and it is protective. Surprisingly, IL-22 deficiency also appeared to result in enlarged cLN in the context of recall *C. albicans* infections, a phenomenon that was not seen during the primary response. This expansion was accompanied by decreased $CD4^+$ T cell proliferation, decreased $CD4^+IFN\gamma^+$ frequencies and elevated $CD4^+IL\text{-}17A^+$ frequencies. Therefore, during the adaptive response to oral *C. albicans* challenge, IL-22 appears to act on the cervical LN (cLN) stroma to modulate CD4 effector function.

5.2 Results

5.2.1 IL-22 is induced during primary and secondary *Candida* infection

We previously developed a model of secondary (recall) oral *Candida* infection to assess the role of the adaptive response in OPC. Using this system, we demonstrated that Th17 cells indeed confer long term protection against OPC [243]. Given the context-dependent role of IL-22 in acute and chronic infection/inflammation models, we sought to determine the role of IL-22 during adaptive (recall) *Candida* infections. *C. albicans* specific IL-22-producing CD4 T cells

have been described in humans but they have not been assessed in mice in the context of OPC [151, 265, 315].

To assess the state of IL-22 expression during secondary OPC, WT mice were subjected to OPC. Six to eight weeks after the primary exposure, mice were orally re-challenged with *C. albicans,* and PBS-inoculated mice were used as negative controls (**Fig. 5.1A**). Tongue was harvested at day 2 post infection and *Il17a* and *Il22* expression was assessed. As previously reported [37, 74, 308], both *Il17a* and *Il22* were induced after primary oral *Candida* infection. Similarly, *Il17a* and *Il22* were upregulated during secondary OPC, although surprisingly there were no statistical differences observed between the primary and secondary infection (**Fig. 5.1B, C)**. To confirm the antigen specificity of responding lymphocytes, cervical lymph node (cLN) cells were harvested at day 3 post re-challenge and stimulated with heat-killed (HK) *C. albicans* for 5 days. IL-17A and IL-22 were assessed in the supernatants by enzyme-linked immunosorbent assay (ELISA). As previously reported, there was a robust expression of IL-17A (~5000 pg/ml) after re-challenge. Mice receiving only a primary infection also expressed IL-17A (~1000 pg/ml) but IL-17A was undetectable in sham-infected mice (**Fig. 5.1D**), as previously described (Hernandez Santos et al., 2013). IL-22 expression followed a similar pattern, with ~2500 pg/ml IL-22 seen during recall challenge and ~1000 pg/ml in mice subjected to only primary infection (**Fig. 5.1E**). Therefore, *C. albicans*-specific IL-17A and IL-22 responses are induced during recall *Candida* infection, although it seemed that there was higher magnitude of cLN IL-17A expression compared to IL-22 during the adaptive response.

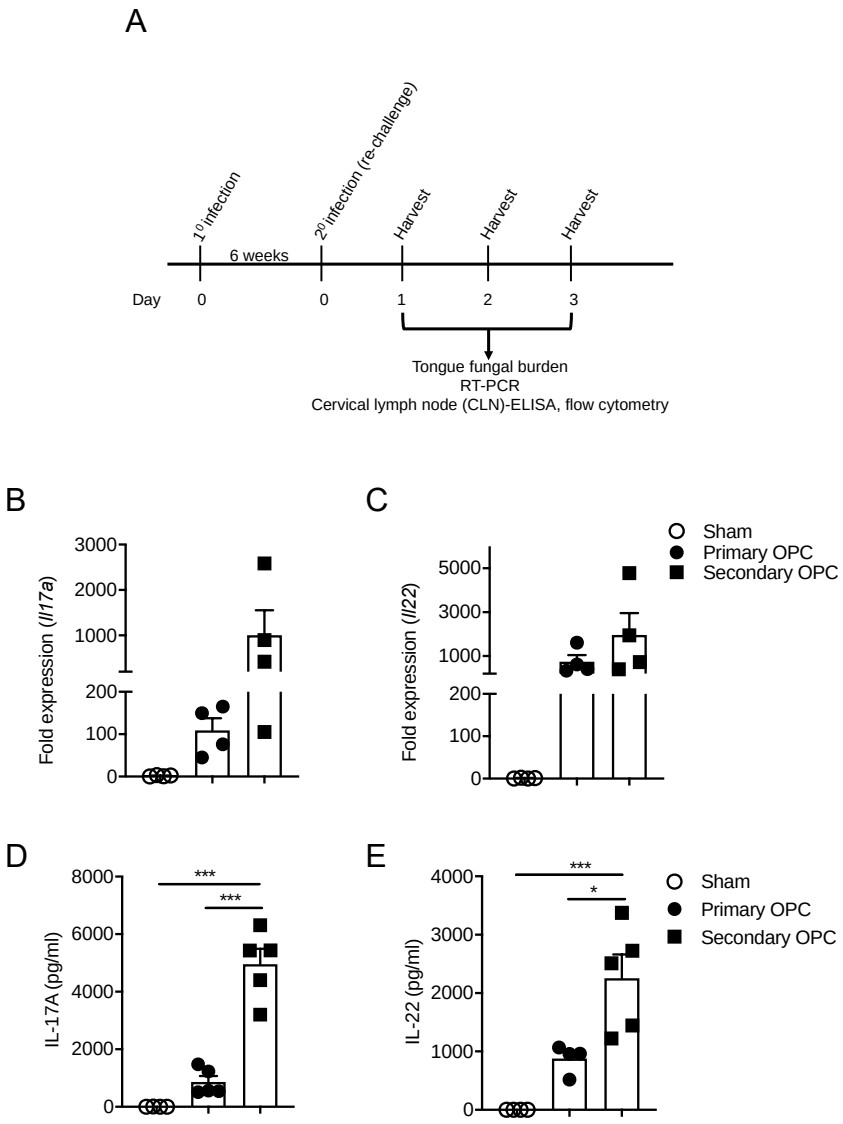

Figure 5.1: *C. albicans* specific cLN lymphocytes express IL-17A and IL-22 during recall OPC

(**A**) Recall OPC infection model diagram showing timelines of infection and harvest. WT mice were orally challenged with *C. albicans* (primary OPC) or phosphate-buffered saline (PBS-sham). Six weeks later, mice were re-infected with *C. albicans* or treated with PBS. Tongue and cervical lymph nodes cLN were harvested at the indicated time points. Total mRNA from day 2 tongue homogenates of infected WT mice was subjected to qPCR for *Il17a* (**B**) and *Il22* (**C**) and normalized to *Gapdh*. CLN cells were harvested at day 3 post primary and secondary OPC and

cultured ± heat killed (HK) *C. albicans* for 5 days. IL-17A (**D**) and IL-22 (**E**) in supernatants were assessed by ELISA. Graphs show mean +SEM and each symbol represents a mouse. Data were analyzed by ANOVA. *p<0.05, ***p<0.001.

5.2.2 IL-22 protects against persistent oral *C. albicans* infection

To assess the functional relevance of IL-22 during the adaptive response to oral *C. albicans* infection, wild type (WT) and *Il22*$^{-/-}$ mice were subjected to a recall OPC model, and fungal burdens were assessed at days 1 to 3 post infection. During both primary and recall OPC, WT mice largely cleared the infection (primary infection average CFU~ 132, recall infection average CFU~ 32) while *Il22*$^{-/-}$ mice still had significantly higher fungal burdens at day 3 p.i. (primary infection average CFU~2964, recall infection CFU~1350) (**Fig. 2A, B**). WT mice that received both primary and recall infections had a slightly lower but statistically insignificant decrease in fungal burden (**Fig. 2B**). A lower percentage of these mice (33.3%) still had detectable fungal burdens at day 3 compared to those receiving only a primary infection, where 50% failed to clear *C. albicans* (**Fig. 2C**). In contrast, *Il22*$^{-/-}$ mice maintained higher fungal burdens and persistent colonization during both primary and recall challenge (**Fig. 2C**). Therefore, prior exposure to *C. albicans* induces a modestly more efficient pathogen clearance in WT mice, while IL-22 deficiency resulted in impaired clearance and persistent colonization.

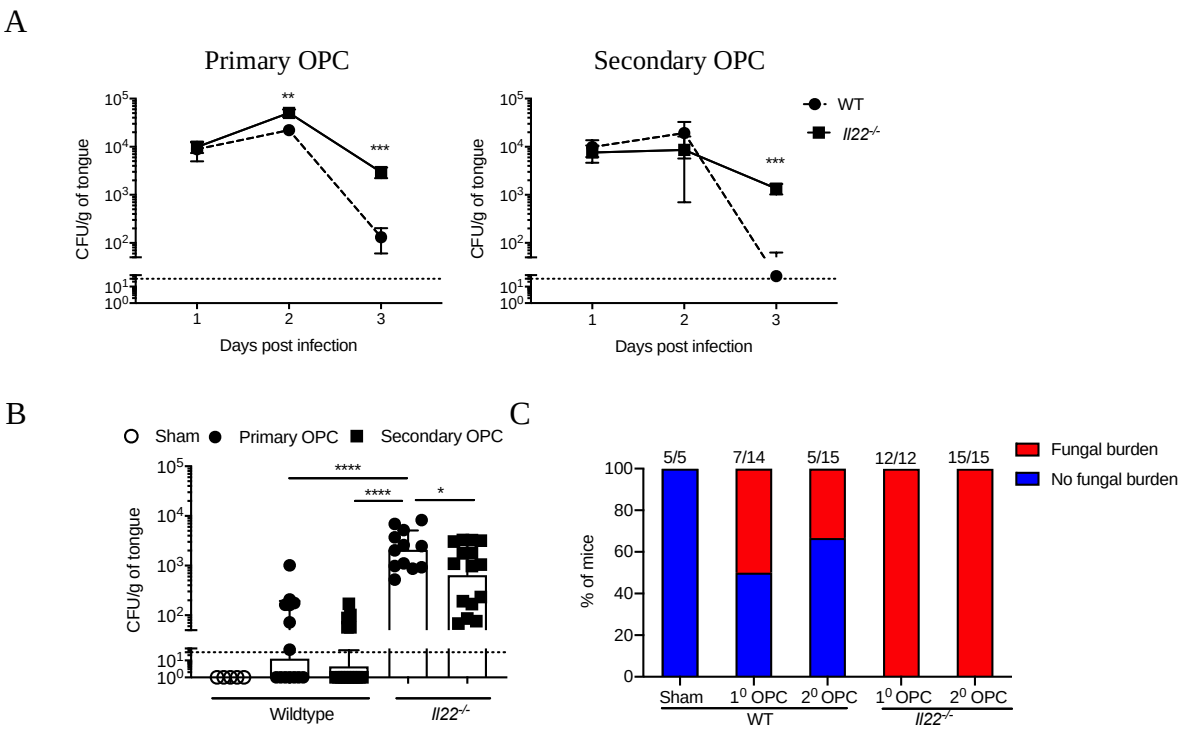

Figure 5.2: IL-22 protects against persistent *C. albicans* colonization

WT and *Il22*$^{-/-}$ mice were subjected to primary and secondary OPC. PBS inoculated group was maintained as sham control. Tongues were harvested on days 1, 2 and 3 p.i. and fungal burdens were assessed by enumerating colony forming units (CFU) of tongue homogenates on agar plates. (A) Data show mean CFU at the indicated time points +SEM for primary OPC (left) and secondary OPC (right). Data is from one experiment with 4-5 mice per group per time point. (**B)** CFUs assessed at day 3 as in panel (A). Bars show geometric mean + SD. Dashed lines represent the limit of detection. Data were pooled from 3 experiments and each symbol represents a mouse. (**C**) Analysis of data from panel B showing the percentage of mice with fungal burden. Data were analyzed by ANOVA, with Mann-Whitney correction for fungal load analysis. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

5.2.3 IL-22 may regulate cLN structure and cLN IL-17 responses during secondary OPC

To assess the nature of the T cell compartment during a recall (adaptive) response to oral *C. albicans* infection, we turned our focus to the draining cLN, a major site of immune activation in response to oral insults. There were few notable differences in cLN from WT sham, WT primary OPC and WT re-challenged mice (**Fig. 5.3A, B**). Unexpectedly, we observed that *Il22*$^{-/-}$ mice had larger cLN than their WT counterparts, and this difference was most prominent in re-infected *Il22*$^{-/-}$ mice (**Fig. 5.3A, B).** Since antigen-specific Th17 cells mediate protection against secondary OPC [129, 243], we first hypothesized that the enlarged cLN observed in *Il22*$^{-/-}$ mice may be due to increased proliferation of CD4 T cells undergoing priming. However, this appeared not to be the case, as cLN CD4 T cells from *Il22*$^{-/-}$ mice had lower proliferative capacity than WT CD4 T cells, as assessed by Ki67 staining (**Fig. 5.3C-E).** Hence the enlarged cLN in *Il22*$^{-/-}$ mice is not likely due to proliferative expansion of CD4 T cells.

This observation led us to evaluate the cytokine output and activation state of cLN T cells, measured at day 3 after either a primary or secondary *C. albicans* infection. While there was no difference in the frequency of $CD4^+$ $IFN\gamma^+$ cells in WT mice regardless of the number of infections, there was a decreased trend for $IFN\gamma^+$ $CD4^+$ cells in *Il22*$^{-/-}$ mice particularly during secondary infection (**Fig. 5.4A, B**). There was a small but statistically significant increase in $CD4^+$ IL-$17A^+$ cells in WT mice during secondary infection (averaging 1.5%) compared to WT and *Il22*$^{-/-}$ mice subjected to only primary infection (**Fig. 5.4A, C**). Unexpectedly, there were even higher frequencies of $CD4^+$ IL-$17A^+$ cells (~ 2.5%) in *Il22*$^{-/-}$ cLN following secondary infection (**Fig. 5.4A, C**). The activation state of $IFN\gamma^+CD4^+$ and $CD4^+$IL-$17A^+$ cells was evaluated by co-staining with CD44, a marker for effector-memory T cells (T_{EM}). While there was little change in the

frequency of $CD4^+CD44^+$ $IFN\gamma^+$ cells in WT mice, there was ~10% decrease in $CD4^+CD44^+$ $IFN\gamma^+$ cell frequency in *Il22*$^{-/-}$ mice during secondary infection (**Fig. 5.5 A, B**). In contrast, the frequency of $CD4^+CD44^+$IL-$17A^+$ cells increased in WT mice (~9%) in response to secondary infection (**Fig. 5.5 A, C**). The absence of IL-22 resulted in even higher frequency of $CD4^+CD44^+$ IL-$17A^+$ cells (~14%) during secondary infection. Together, these data suggest that IL-22 deficiency promotes effector Th17 activity during secondary OPC.

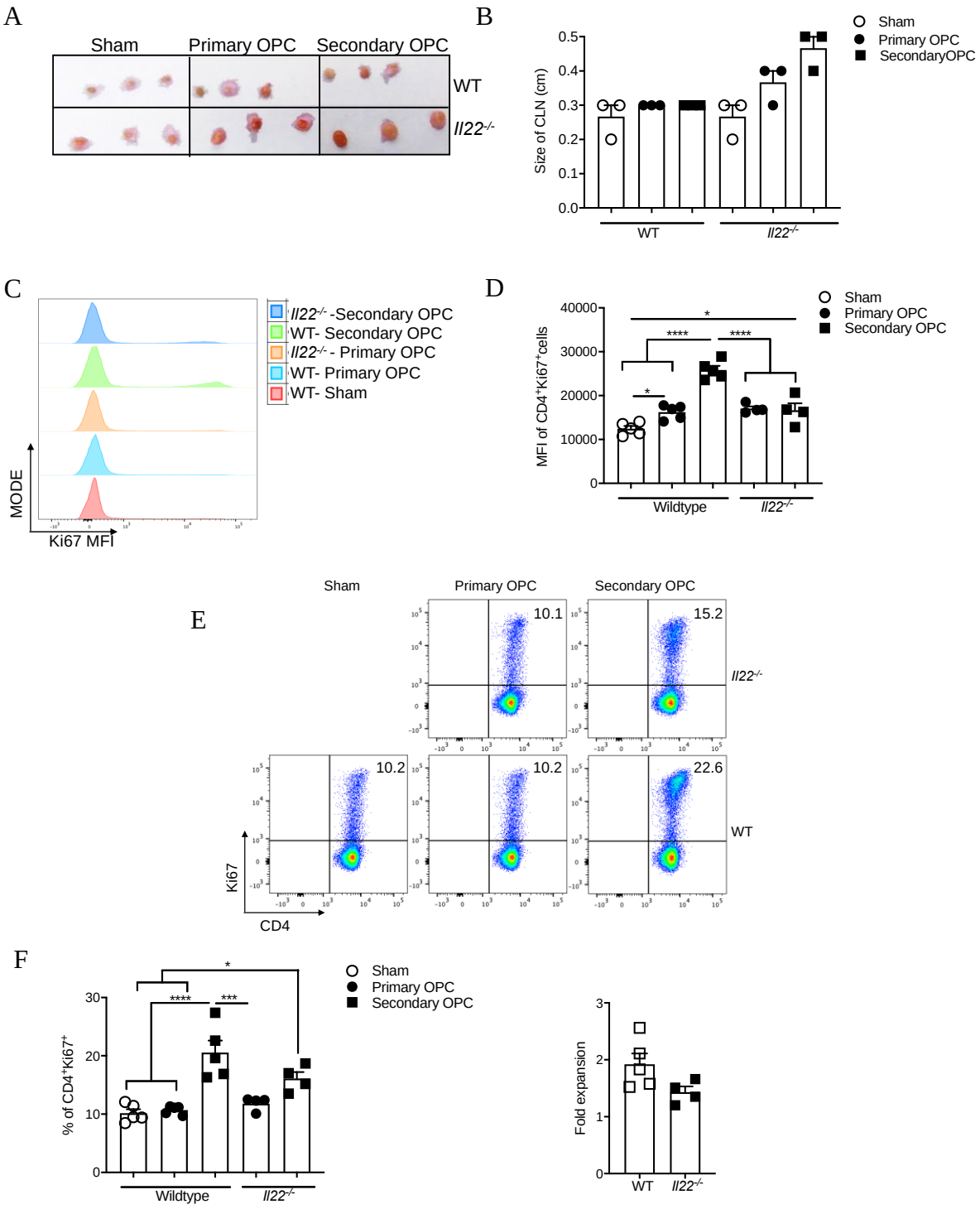

Figure 5.3: *Il22* deficiency increases cLN size and impairs CD4 proliferation during reinfection

WT and *Il22*$^{-/-}$ mice were subjected to primary and secondary OPC. PBS inoculated group was maintained as sham control. (**A**) Diagram and (**B**) summary plot showing the increase in cLN size at day 3 post infection. Data is representative of 3 independent experiments. Harvested cLN cells were stained and gated on live TCRB^{+}CD4^{+}Ki67^{+} cells. Representative histogram (**C**) and (**D**) summary plot showing Ki67 MFI in the treatment groups. (**E**) Representative FACS plot showing Ki67 staining as in (**C)** and (**D)**. (**F**) Left; Summary plot and right; fold expansion of TCRB^{+}CD4^{+}Ki67^{+} cells in WT and *Il22*$^{-/-}$ mice during reinfection. Data are from 1 experiment. Each symbol represents a mouse. Data were analyzed by ANOVA *p<0.05, ****p<0.0001.

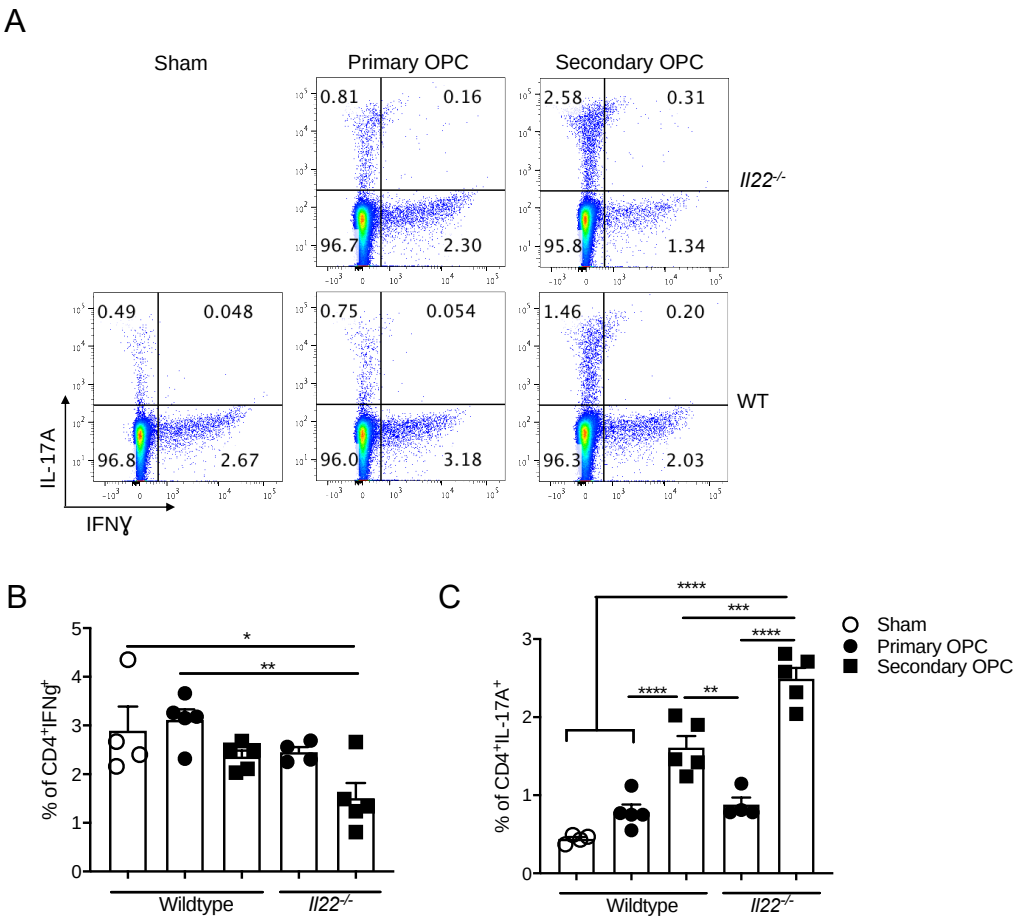

Figure 5.4: IL-22 modulates CD4IFN-γ$^+$ and CD4IL-17A$^+$ cell frequency during secondary OPC

WT and *Il22*$^{-/-}$ mice were subjected to primary and secondary OPC. PBS inoculated group was maintained as sham control. Harvested cLN cells were stained and gated on live TCRB$^+$CD4$^+$IL17A$^+$ and TCRB$^+$CD4$^+$IFNγ$^+$ cells. (**A**) Representative FACS plot (**B**). Quantification of TCRB$^+$CD4$^+$IFNγ$^+$ cells and (**C**) TCRB$^+$CD4$^+$IL17A$^+$ from A. Data is from 1 experiment and each symbol represents a mouse. Data were analyzed by ANOVA *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

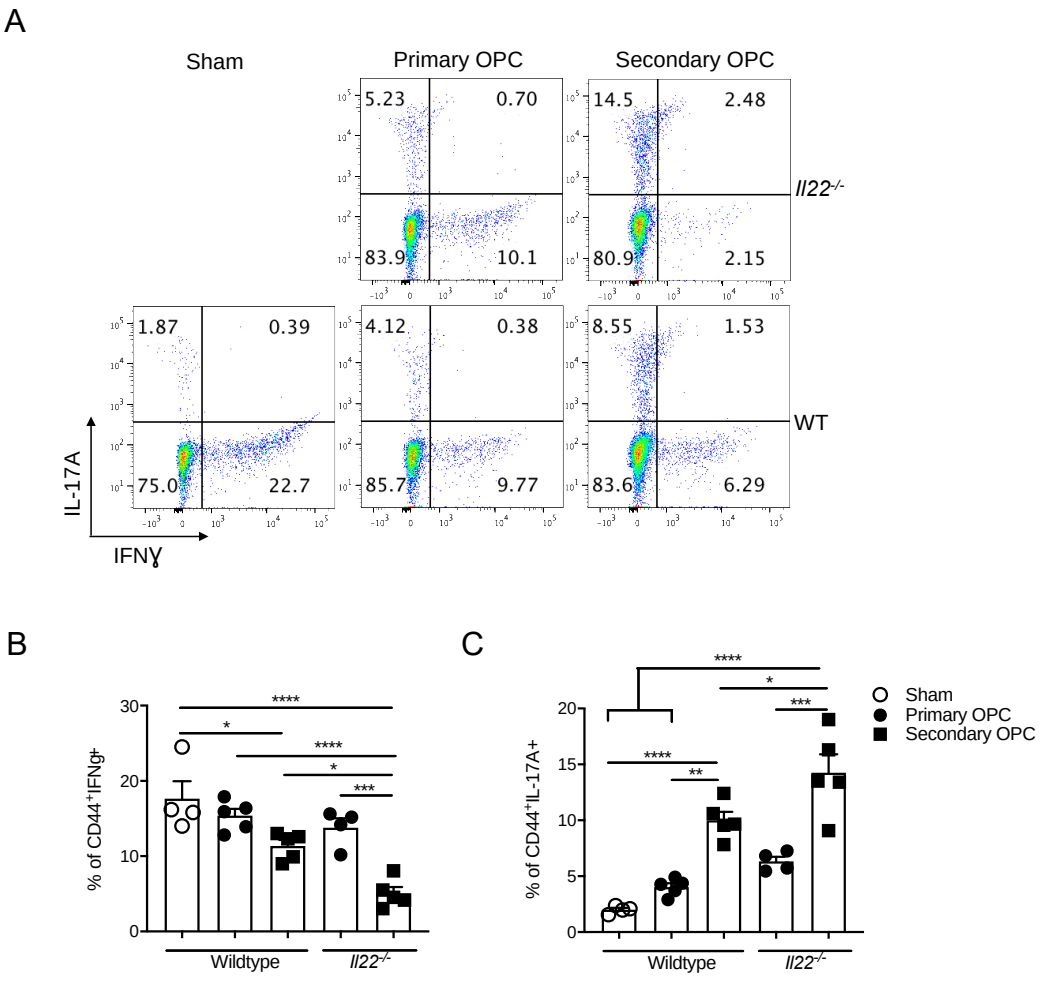

Figure 5.5: IL-22 modulates the activation state of CD4IFN-γ$^+$ and CD4IL-17A$^+$ cells during secondary OPC

WT and *Il22*$^{-/-}$ mice were subjected to primary and secondary OPC. PBS inoculated group was maintained as sham control. Harvested cLN cells were stained and gated on live TCRB$^+$CD4$^+$CD44$^+$IL17A$^+$ and TCRB$^+$CD4$^+$CD44$^+$IFNγ$^+$ cells. (A) Representative FACS plot (B). Quantification of TCRB$^+$CD4$^+$CD44$^+$IFNγ$^+$ cells and (C) TCRB$^+$CD4$^+$CD44$^+$IL17A$^+$ from A. Data is from 1 experiment and each symbol represents a mouse. Data were analyzed by ANOVA *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

5.3 Discussion

Unlike humans, mice are naïve to *C. albicans* and provide a suitable experimental system to interrogate the roles of IL-22 during innate and adaptive response to oral *C. albicans* infection. In this study, I sought to understand the role of IL-22 during recall (adaptive) responses to oral thrush. Both, IL-17A and IL-22 were induced in murine tongue during the innate and adaptive response to OPC (**Fig. 5.1**) [37, 53, 74, 243, 308]. Robust *C. albicans*-specific IL-22 and IL-17 expression was observed when cLN cells were co-cultured with HK *Candida* under recall settings. Thus IL-22 responses are locally induced in the tongue as well as in the peripheral draining cLN during adaptive response to oral *C. albicans* challenge. Other studies have demonstrated recall IL-22 responses when peripheral blood from healthy donors were stimulated with HK *Candida* [151, 265, 315]. Recall IL-22 responses are therefore present in humans and generated in mice in response to experimental *C. albicans* infection.

Conventional Th17 cells generated during the adaptive response do appear to augment the innate Type 17 response to promote antifungal immunity, as would be expected for an adaptive response. Pre-exposure to oral *C. albicans* challenge modestly enhanced clearance and reduced prolonged colonization (**Fig. 2. A-C**) [53, 243]. However, *Il22*$^{-/-}$ mice had sustained fungal burdens and persistent colonization regardless of previous encounter with *C. albicans* (**Fig. 2. A-C**). In the current experimental set up, it is hard to tell whether the observed fungal burden during the recall challenge is a failure of adaptive IL-22 mediated responses or a carry-over effect of *Il22* deficiency from the innate response. As noted previously, mice mount a robust innate Type 17 response against primary *C. albicans* exposure [74, 243]. Since the innate response drives the unset of adaptive immunity, it is conceivable that the failure to clear *C. albicans* is due to the accrued loss of IL-22 driven protection from the primary and secondary exposure.

Innate Type 17 cells (mainly TCRβ^{+}and TCRγδ^{+} cells) are the sources of IL-17A, IL-17F and IL-22 during primary OPC [86, 308, 316]. Surprisingly, both *TCRβ*$^{-/-}$ and *TCRγδ*$^{-/-}$ mice are resistant to OPC while *Rag*$^{-/-}$ mice are susceptible [74, 242]. Similarly, CD4 but not CD8 T cells are the dominant source of IL-17 during adaptive responses to oral *C. albicans* challenge. However, *CD4*$^{-/-}$ and *CD8*$^{-/-}$ mice are resistant to OPC. These outcomes are attributed to compensatory mechanisms during the primary genetic deficiency. In recall *C. albicans* infection in WT mice, CD4 but not CD8 T cells are the dominant source of IL-17A. However, in *C. albicans* rechallenged CD4 or CD8 deficient mice, there was a comparable increase in CD8IL-17A^{+} and CD4IL-17A+ cells respectively [243]. Although the consequence of *Il17a* deficiency in the adaptive response to *C. albicans* has not been assessed, it is remarkable that *Il22* deficiency by itself impairs antifungal immunity during both primary and secondary exposure to oral *C. albicans* challenge.

Immune cell infiltration and translocation of *C. albicans* to the cLN could hypothetically have accounted for increased cLN size observed in *Il22*$^{-/-}$ mice during re-infection. However, upon culturing cLN cells with HK candida in medium lacking any antifungal drug, there was no evidence of hyphal growth or cloudiness in the media (data not shown). This observation suggested that the enlarged lymph node could not be due to translocation of live *Candida* to the cLN. Although the total numbers of immune cells infiltrating the cLN were not assessed in this experiment, the decreased proliferative capacity of CD4 T cells from *Il22*$^{-/-}$ mice (**Fig. 5.3 C-F**) during reinfection indicates that the increased cLN size is not likely due to aberrant CD4 T cell expansion. Instead, IL-22 may be acting on the cLN stroma during *C. albicans* reinfection to modulate the cLN stroma and promote CD4 T cell expansion. In contrast to the impaired CD4 proliferation observed in *Il22*$^{-/-}$ mice during *C. albicans* reinfection, IL-22 exerted opposing

modulatory effects on cLN CD4 IFNγ and IL-17A cell frequencies. While the frequency of IL-17A$^+$ CD4 T cells and CD44$^+$CD4$^+$IL-17A$^+$ cells were elevated in *Il22*$^{-/-}$ mice during reinfection, CD44$^+$CD4$^+$IFNγ$^+$ cells were decreased. It therefore appears that IL-22 acts to dampen cLN IL-17A output during *C. albicans* reinfection while promoting IFNγ responses. A previous report showed increased *Il17a* and *IFNγ* transcripts in *Il22*$^{-/-}$ mice in a gastric *C. albicans* infection model. Here, it was suggested that IL-22 exerts reciprocal regulation of IL-17A and IFN-γ [238]. While there are major differences between the gastric candidiasis and OPC model, it appears that some common facets of IL-22 mediated responses may apply to both settings.

Our understanding of Th17 memory cells is still evolving. Th17 cells are plastic and frequently interconvert between Th1 and Treg phenotypes [254, 317]. Using IL-17 fate tracker mice, the capacity of Th17 cells to convert into Th1 cells and dual expressing IL-17A$^+$IFNγ$^+$ cells was demonstrated in the setting of chronic autoimmunity. However, this switching did not occur in acute dermal candidiasis, which is γδ-T cell predominated [254]. In our hands, very few IL-17A$^+$IFNγ$^+$ CD4 T cells were present in WT or *Il22*$^{-/-}$ mice during reinfection, suggesting that there is limited plasticity in the Th1 and Th17 populations. Moreover, the opposing effects of IL-22 deficiency on IL-17A and IFN-γ was observed only during reinfection. IFN-γ is documented to inhibit Th17 differentiation through enhanced STAT1 activation [318, 319]. It is therefore likely that IL-22 may be modulating cLN Th17 responses through IFN-γ-expressing Th1 cells. Although Th17 cells and IL-17RA signaling are important in protecting against oral thrush [74, 86, 243], exuberant Th17 responses may also promote inflammation and tissue pathology.

Regardless of these opposing effects of IL-22, our data so far suggest that during oral *C. albicans* recall infection, IL-22 modulates T cell responses and the cLN environment. Although IL-22RA1 expression is reported to be restricted to cells of non-hematopoietic origin, IL-22RA1

expression has been validated on thymic epithelial cells during thymic injury [140] and on lymph node stromal cells in a model of collagen-induced arthritis (CIA) [313]. In the CIA model, IL-22RA1 signaling on the LN stroma promoted germinal center reactions [313]. In a related study, it was demonstrated that the lymphoid stroma inhibits activated T cell proliferation [311].

Interestingly, the lymph node stroma also modulates sphingosine 1 phosphate (S1P)/ sphingosine 1 phosphate receptor (S1PR) signaling to control emigration of activated T cells from the lymph node [310, 320]. Therefore, a plausible alternative explanation for the increased frequency of Th17 responses and cLN hypertrophy in the absence of IL-22 could be due to the failure of Th17 cells to egress from the cLN into the local tongue tissue to exert antifungal immunity. IL-22 signaling modulates the sphingosine1 (S1P) sphingosine receptor axis (SIPR) in metastatic cancer to promote macrophage infiltration [321]. The observations in our study will therefore be consistent with a speculation that IL-22 acts on the cLN stroma during adaptive oral *C. albicans* infection to modulate T cell effector functions and egress. Although germinal center responses were not assessed in these experiments, a related study in experimental autoimmune encephalitis (EAE) showed that, while IL-17 signaling in fibroblastic reticular cells of the lymph node was not required for Th17 development, it was important for germinal center formation and antibody production [309].

To date, there are no licensed antifungal vaccines, but an experimental vaccine for *C. albicans* is in development. NDV-3, a recombinant alum conjugated vaccine based on the fungal cell wall invasion protein agglutinin-like sequence 3 (Als3p) is the most clinically advanced. In phase 1 clinical trials, NDV-3 was well tolerated and induced antigen specific production of IL-17A and IFN-γ [322]. NDV3 also elicited moderate protection in animal models of oropharyngeal, vaginal and invasive candidiasis through T and

B cell mediated responses [323-325]. Moreover, this vaccine was cross protective against *Staphylococcus aureus* (*S. aureus*) infection due to high structural homology between Als3p and *S. aureus* clumping factor-A. Since recipients of fungal vaccines are likely to be persons with underlining immunocompromised conditions, it is imperative to identify correlates of protection and their underlying mechanism(s) of action in order to boost appropriate immunity to the extent possible. The findings of this study reveal perturbations in the Th17 response and lymph node structure in the absence of IL-22 and open new questions to understand how IL-22 and IL-17 collectively shape the adaptive response to oral thrush.

6.0 Conclusions and Future Directions

6.1 Summary

In this dissertation, I expanded the field's understanding of the mechanisms underlying IL-22 mediated protection in the setting of acute OPC (innate response) and in response to recall/secondary OPC (adaptive response). In chapter 3, I found that IL-22 is non-redundant with IL-17RA signaling in mediating protection against OPC. Among their key differences in driving antifungal events was the finding that IL-22 and IL-17RA signaling exerted opposing effects on neutrophil recruitment. While IL-17RA signaling promotes neutrophil recruitment, IL-22 dampens neutrophil recruitment. Moreover, IL-22RA1 was found to be expressed in an anatomically distinct region from IL-17RA in the stratified oral epithelium. While IL-17R is required in the SEL to protect against OPC, IL-22RA1 is required in the underlying BEL. IL-22 is required to drive proliferation of BEL cells which in turn give rise to the differentiated SEL cells which express IL-17RA. Together, these data support the spatially distinct roles of IL-17RA and IL-22RA1 during OPC and show that IL-22 signaling in the BEL promotes the replenishment of the sloughed SEL and hence licenses IL-17R signaling in the SEL to drive antifungal immunity in the oral mucosa.

In chapter 4, I identified an additional role of STAT3 in oral thrush besides its known roles of promoting protective Th17 responses. I found that STAT3 signaling in the BEL is required for protective oral antifungal immunity. STAT3 was identified as a key transcriptional hub integrating IL-22 and IL-17RA signaling networks. Notable among these targets are IκBζ, C/EBPβ, Fos, JUNB, MYC, BCL3, the RNA binding protein ZFP36 (better known as tristetraprolin) and the receptors Il1RN and CXCR3. Thus, in addition to promoting differentiation and maintenance of

Th17 cells, BEL STAT3 co-ordinates antifungal immunity by targeting key transcriptional events in the responding or *C. albicans* invaded epithelial cells.

In chapter 5, I assessed the role of IL-22 in recall/adaptive OPC setting. *Il22*$^{-/-}$ mice are sensitive to secondary OPC although it remains to be determined whether their sensitivity is due to accrued defects from the primary exposure. Regardless of this caveat, IL-22 deficiency in recall OPC setting resulted in increased cLN size and weight despite decreased proliferation of cLN CD4 cells. Moreover, IL-22 deficiency promoted IL-17A Th17 effector activity while hampering IFN-γ responses. These data highlight potential differences in the role of IL-22 in the oral mucosa and the cLN during acute and recall oral *C. albicans* exposure. In recall *C. albicans* exposure settings, IL-22 modulates cLN structure and effector CD4 responses. These findings support a model where IL-22 signaling in the cLN stroma likely modulates cLN structure and organization and effector CD4 T cell responses and possibly egress during OPC.

Together, these findings bring to light many previously unappreciated mechanisms of oral antifungal immunity and point to the co-ordination among key cytokines, immune cells, oral epithelial cells and transcription factors to elicit appropriately toned protective antifungal immune responses. Although IL-22 and IL-17RA signaling work in concert to drive oral antifungal immunity, there are some notable differences in in their mechanisms of action, localization of their responding receptors in the oral epithelium as well as the downstream signaling pathways they activate. Even more revealing is how signaling through their disparate receptors is integrated in the responding epithelial cells to mount effective antifungal immunity. Additionally, we identified a role for IL-22 in cLN during adaptive *C. albicans* response partly through elevated IL-17A response. This finding has implications for understanding the roles of IL-22 on cLN stroma and how it shapes effector T cell responses.

6.2 Unifying Discussion, Outstanding Questions and Future Directions

Our current and previous findings on the activation requirements for IL-17 and IL-22 production in OPC reveal important and intricate roles for candidalysin and IL-23. While the role of IL-23 in maintaining effector functions of conventional Th17 cells is well known, the mechanisms by which IL-23 activates IL-17 and IL-22 production from innate immune cells in OPC (mainly TCRβ$^+$ and γδT T cells, as shown here) is less understood. In conventional Th17 cells, IL-23 induces STAT3 activation and upregulation of RORγT to promote Th17 effector functions and maintenance [97]. However, deletion of STAT3 in CD4 T cells did not exacerbate susceptibility to OPC [86]. Candidalysin promotes innate TCRβ$^+$ cell expansion and *Il17* induction during OPC [37]. In our current study, candidalysin was also important for IL-22 expression during OPC. It is thought that candidalysin triggered IL-1 signaling from the hematopoietic and stromal compartments account for this observation [37]. However, the direct mechanisms of how candidalysin induces TCRβ and γδT cell activation is still unclear. γδT cells and tissue resident CD4 T cells have been described in many mucosal tissues where they are poised to respond quickly to insults and injury [326, 327]. Similar responses are important for eliciting a fast response to oral *C. albicans* infection, and it will be interesting to understand more about how candidalysin activates γδT cells and oral resident CD4$^+$ T cells during OPC. In this regard, EGFR was recently determined to be required for candidalysin mediated signaling on epithelial cells [36]. EGFR is expressed in immune cells [328-330] and it will be important to determine whether candidalysin-mediated EGFR signaling on γδT$^+$ and TCRβ$^+$ cells directly contributes to Type17 cell responses in OPC.

Beyond candidalysin, there is likely to be more roles for DAMPs released during *Candida* hyphal invasion in the activation of "Type 17" cells. Hyphal invasion induces damage to the epithelium leading to apoptotic and necrotic cell death [331]. Necrosis can lead to the release of various DAMPs including mitochondrial DNA, N-formyl peptides, cardiolipin, cytochrome C, carbamoyl-phosphate synthase 1, ATP and calreticulin which can have varied effects on inflammation and effector cell responses [332-336]. Particularly, ATP signaling through P2X7R promotes inflammasome activation leading to the production of IL-1β which is necessary for Th17 differentiation [267, 337]. Similarly, calreticulin promotes Th17 differentiation through enhanced IL-6 and TNF-α secretion [336]. Hence, there is a likely role for DAMPs in the activation of "Type 17" immunity during OPC although most of these are yet to be experimentally determined.

In determining the cellular sources of IL-22 during OPC, we found that γδT cells but not TCRβ$^+$ cells were the dominant source of IL-22, which contrasts somewhat with our previous observations regarding sources of IL-17A. Although one study suggested the presence of IL-17 producing ILCs in OPC [117], it is clear that the dominant cellular sources of IL-17 and IL-22 in OPC are TCRβ$^+$ and γδT cells, respectively. While it is likely that dual producers of IL-17 and IL-22 will exist in these populations, technical challenges in obtaining viable cells for flow cytometry from this tissue prevented us from assessing this. Our current isolation protocol coupled with the use of IL-17 and IL-22 dual reporter mice will be useful in fully delineating the cellular sources of IL-17 and IL-22. However, the direct assessment of the functional relevance of IL-17 and IL-22 producing TCRβ$^+$ and γδT cells in OPC is also challenging because of the very low proportions of cells that can be isolated from the tongue, making it impossible to do adoptive transfer studies. Moreover, both *TCRβ*$^{-/-}$ and *TCRγδ*$^{-/-}$ mice are resistant to OPC, although *Rag1*$^{-/-}$ mice are sensitive. Hence there is a likely compensatory mechanism at play [86, 243]. Thus, while dual

producers of IL-17 and IL-22 can be assessed in future studies, it is not very likely that the functional relevance of IL-17 and IL-22 producing TCRβ^+ and $\gamma\delta$T cells in OPC can be directly assessed.

Mucosal barriers are uniquely adapted to provide and support immune responses in their respective niches. Compared to other barrier sites like the skin, gut and lung, the mucosal immunosurveillance networks in the oral cavity are poorly understood. The oral cavity has niches such as the tongue, gingiva, buccal mucosa, tonsils and hard palate that are adapted to limit excessive inflammation, promote tissue repair and maintain the oral microbiome. Findings from our current study show that, unlike IL-17RA [88], IL-22RA1 is localized and required in the BEL to promote oral antifungal immunity.

This discovery opens up opportunities to interrogate the functional consequences of IL-22RA1 signaling in the BEL. An unanswered question is whether early oral exposure to *C. albicans* in humans (for example, in vaginal delivery) confers any adaptations to the oral epithelium that enable it to better deal with later assaults in life. This early exposure occurs long before introduction to solid food which likely causes masticatory stress to the oral cavity [338]. In this regard, a recent seminal paper demonstrated that prior inflammatory assault or *C. albicans* infection in the skin confers epithelial stem cell memory in K14$^+$ cells to respond better to subsequent wounding [270], but it is unknown whether something similar occurs in the oral epithelium.

Moreover, the BEL is home to stem cells but comparatively little is known about how they impact oral immunity [81, 82]. Lrg1$^+$ and Lrg5$^+$ stem cells have been described in the hard palate and dorsum of tongue respectively [77, 79]. In the hard palate, Lrg1 stem cells maintain quiescence during homeostasis but lose their dormancy in response to wounding and masticatory stress [77].

Whether stem cells in the oral cavity express IL-22RA1 or not and their role in oral antifungal immunity and oral mucosal immunity in general is not known.

In addition to contributing to oral antifungal immunity, IL-22/IL-22RA1 in the oral BEL may impact other aspects of oral immunity including oral malignancies. Oral squamous cell carcinoma (OSCC), which mostly occurs on the tongue and floor of the mouth, is a common manifestation in head and neck cancer patients with poor prognosis [339]. Chemotherapy and radiotherapy in these patients are often complicated by increased risks of oral and gastrointestinal candidiasis and candidemia [340-342]. This complication is partly driven through epithelial interactions with *Candida*. Models of oral cancer target mutations in basal epithelial cells to induce malignant transformation [339]. The role of IL-22RA1/STAT3 in maintaining stemness in other cancers including pancreatic cancer, colon cancer, have been described [343]. The localization of IL-22RA1 in the oral BEL suggests a potential role for IL-22RA1 expressing stem cells in OPC. This finding may be translatable to the roles of IL-22RAI in $Lrg5^{+}$ stem cells in the intestinal epithelium, as were shown in a recent study. In one report, IL-22RA1 signaling in intestinal stem cells protects against colon cancer incidence and genotoxic stress [216]. Thus, assessing the role of BEL IL-22RA1 in OSCC and oral genotoxic stress will be revealing. Whether oral BEL IL-22RA1/STAT3 has roles in oral malignant transformation and OSCC is not appreciated. Our current findings of the role of BEL IL-22RA1 and STAT3 provide further impetus to understand how these pathways could influence oral malignancy. By subjecting $Il22ra1K14^{Ert2Cre}$, $Il22ra1Lrg5^{Ert2Cre,}$ $STAT3K14^{Ert2Cre}$ and $STAT3Lrg5^{Ert2Cre}$ mice to radiation induced mucositis and OPC, some of these pertinent questions can be addressed.

In view of the masticatory stress, constant damage, exposure to food allergens and microbes encountered in the oral cavity, it is conceivable that the stratification and spatial

distribution of IL-17RA and IL-22RA1 may serve broader functions to maintain homeostasis in oral immunity. However, our understanding of the relationships between the cells in the stratified oral epithelium is still very rudimentary. The findings made in this dissertation can help lay the foundation for deeper investigations. Newly available tools such as spatial transcriptomics will be valuable for the assessment of the relationships in the various cell types present in the oral epithelium while providing critical information on their transcriptional profiles.

The impact of good oral health on general well-being has been long appreciated. Degeneration of oral health as in oral thrush and periodontitis are associated with worsened outcomes in diabetes, gut inflammatory conditions and cardiac health [3, 344, 345]. A recent publication highlighted the intramucosal connection between the oral cavity and the gut in commensal pathobiont driven colitis. Oral microbes and pathobiont reactive Th17 cells generated in the cLN in response to periodontitis translocate to the gut to exacerbate gut inflammation [345]. This current study and previous findings highlight the importance of IL-17RA and IL-22RA1 signaling in the oral mucosa in protecting against both oral thrush and periodontitis [3, 74, 86, 308]. However, studies on how the oral immunosurveillance networks driven by OEC IL-17RA-IL-22RA1 lead to oral dysbiosis are lacking. Moreover, it is not clear whether abrogation of IL-17RA and IL-22RA1 signaling in OECs leads to altered translocation of *Candida* to the gut. Whether Th17 cells generated in response to recall OPC in the cLN translocate to the gut to mediate extra-oral functions is not appreciated either. In both mice and humans, cross protective *C. albicans* specific Th17 cells are expanded during intestinal inflammation, presenting increased risk for pulmonary infections [151, 265, 266]. Those studies suggest that breakdown in oral epithelial immunosurveillance networks can potentially induce *C. albicans*-mediated dysbiosis and gut inflammation. Studies to investigate this have been lacking partly due to an absence of

adequate systems to study the oral mucosa. However, findings from our study and currently available tools (*I17raK13^{Cre}*, *Il22ra1K14^{Ert2Cre}*, *I17raK13^{Cre}* x *Il22ra1K14^{Ert2Cre} and Il22ra1$^{-/-}$x Il17ra$^{-/-}$*) will allow these pertinent questions to be addressed.

Data from our transcriptomic analysis of *Il22$^{-/-}$* and *Il17ra$^{-/-}$* mice shed light on the genes and pathways employed by the host to prevent or limit OPC. The central role of STAT3 in the transcriptional hub (**Fig. 4.2**) and the subsequent demonstration of its requirement in the BEL was unexpected since previous studies have relied on the role of STAT3 in Th17 cells to explain the disease in HIES patients (**Fig. 4.4**). Our observation raises many questions. Paramount among them is what are the targets of BEL STAT3 and their role in oral antifungal immunity? Our bioinformatic analyses suggest that STAT3 may be integrating signals from IL-17RA signaling and other pathways to promote optimum antifungal immunity. In this transcriptional network, STAT3 is connected with IκBζ, a key IL-17-induced transcription factor important for induction of IL-17 target genes [125, 126]. Although not carefully defined in OPC, studies from other models show the link between epithelial STAT3 and IκBζ. For example, a model of Sjögren's syndrome showed that disruption of IκBζ induction in lacrimal basal epithelial cells exacerbates disease. Importantly, expression of IκBζ was dependent on K5-driven STAT3 [304]. Analysis of the IκBζ promotor activity showed that, while IL-17 does not activate STAT3, STAT3 is required for IκBζ promoter activity and induction of *Defb4* (BD2, the human orthologue of mouse BD3) in HaCat cells (a human skin keratinocyte cell line) [346]. Similarly, IL-36-induced IκBζ in HaCat cells required STAT3 activity and is linked to impaired induction of some canonical IL-17 target genes [284]. The role of IκBζ in OPC is not clearly defined, and it is not known whether IκBζ is targeted by BEL STAT3. The roles and targets of STAT3 in BEL can further be inferred by analyzing BEL cells from *K14Cre$^{ERT2YFP+/-}$STAT3$^{fl/fl}$* mice and *K14Cre$^{ERT2YFP-/-}$STAT3$^{fl/fl}$* by RNASeq, ChIPSeq

and ATACSeq. Through these analyses, the central role of STAT3 can be further deconvoluted and the targets of oral epithelial STAT3 in OPC can be more clearly defined. By comparing these targets with the known IL-17RA transcriptomic data in OPC, there will be more clarity on how IL-22 driven STAT3 activation links with IL-17RA transcriptional network in OPC. Moreover, through ATACSeq analysis, epigenetic marks induced by STAT3 on target genes can be identified. This analysis will help us to understand whether STAT3 likely mediates epigenetic changes and chromatin accessibility of target genes besides the transcriptional regulation.

There is a dynamic reciprocal balance between STAT3 and STAT1 signaling in many settings, leading to switches from proliferative to apoptotic or from inflammatory to anti-inflammatory responses [184, 347]. For example, STAT1 gain of function mutations are associated with decreased STAT3 activity in CD4 cells, and the inverse is observed in STAT3 gain of function mutations [101-103]. The ultimate cellular response is therefore dependent on the cross talk between these transcription factors, which is regulated by environmental cytokines, the duration of stimulation, and the magnitude of activation [184, 347]. Thus, it will be important to know whether deletion of BEL STAT3 results in any aberration in STAT1 mediated responses during OPC. The mice created in this project (*STAT3K14*^{ERT2} mice) will be a useful system in which to accomplish this.

In addition to the transcriptional roles of STAT3, the pleotropic and even sometimes contrasting effects of STAT3 under physiological and pathological conditions can also be dictated by its subcellular localization and interactions with other signaling intermediates. Fungal invasion into the epithelium induces cellular stress, causing changes in membrane permeability, decreased mitochondrial fitness, increased generation of intracellular ROS, decreased intracellular ATP and calcium influx which involve the mitochondria. Mitochondrial STAT3 is known to be important

in redox homeostasis and ROS generation [348, 349]. STAT3 S727 phosphorylation is known to modulate mitochondrial activity and bioenergetics [347]. Hence, whether STAT3 deficiency in the BEL affects organelles such as mitochondria or endoplasmic reticulum during OPC to respond to the ongoing stress is an outstanding question.

As discussed in Chapter five, *Il22* deficiency during recall/adaptive response to OPC appeared to result in visible changes in cLN size and associated effector $CD4^+$T cell functions. The factors accounting for this observation are not known but there are two obvious hypotheses (1) IL-22 generated in the cLN during OPC may act on the lymph node stroma to alter T cell responses, and/or (2) IL-22 signaling may drive egress of activated T cells from the cLN to the tongue. Supporting these ideas, IL-22RA1 is expressed in stromal cells in the germinal center (GC) of the B-cell follicle during collagen induced arthritis [313]. It is yet to be determined whether IL-22RA1 is expressed in the cLN in recall OPC settings and whether specific deletion of IL-22RA1 from the cLN stroma recapitulates the responses seen in *Il22*$^{-/-}$ or *Il22ra1*$^{-/-}$ mice during adaptive T cell responses. Answering these questions will also allow for a more comprehensive assessment of changes in the cLN stromal and hematopoietic compartments. Lymph node hypertrophy in *Il22*$^{-/-}$ mice could also be due to differential expression of lymphocyte retention and egress markers. Our bioinformatic analysis revealed increased CXCR3 expression in *Il22*$^{-/-}$ mice, which could potentially dampen Th17 recruitment to the tongue (**Fig. 4.1C**). A recent study showed that CXCR3 expression enhances CD8 T cell recruitment to the gut during HIV infection while impairing Th17 homing via the CCR6/ CCL20 axis [350]. Hence, it will be informative to immunophenotype the T cell infiltrate in the tongue and cLN to assess differences between effector T cells and expression of homing markers. Differences in the expression of CXCR3 may explain the impaired migration of Th17 cells from the cLN to the tongue.

In addition to the above hypothesis, an obvious line of investigation is to determine how IL-22 deficiency in adaptive OPC settings impacts on tissue resident memory T cells and the overall nature of the *Candida* specific T cell response. Both IL-17 and IL-22 induce factors including *Ccl20* and *Defb3* which ligate to CCR6 and potentially promote recruitment of Th17 cells [351]. Given that "Type 17" cytokines induce local tissue-specific profiles that can influence recruitment, differentiation, homeostasis and protective functions of effector and memory T cells [352], it will be interesting to determine whether IL-22 deficiency distorts local responses that are necessary for generation and retention of tissue resident memory cells in the oral mucosa in adaptive OPC settings. Some of the currently available tools such as OVA-expressing *C. albicans*, pALS bearing MHC II tetramers and pALS-specific T cells from T cell receptor (TCR) transgenic mice can be used to address this question [244, 353]. For example, adoptive transfer of OVA-specific T cells into WT and *Il22*$^{-/-}$ mice followed by infection with OVA-expressing *C. albicans* will allow for the characterization of (1) *C. albicans* induced memory T cells in the tongue and cLN and (2) IL-22 mediated local effects on the memory T cells at these sites. Secondly, by labeling OVA-specific T cells with tracking dyes, the spatial localization of *Candida* specific T cells relative to other immune cells and stromal cells of the oral mucosa and cLN can be assessed by imaging studies. Through this study, interactions of *Candida* specific T cells with their local environment and the potential distortions in the absence of IL-22 can be captured. Moreover, using pALS bearing MHC II tetramers, *C. albicans* specific responses can be characterized *ex vivo*. This study can be complemented with the use of pALS-specific TCR transgenic mice to further analyze the CD4 mediated memory response to *C. albicans*.

Despite the dominant role of IL-17/IL-17RA signaling, this study suggests that effective oral antifungal immunity involves multiple coordinated signals, and thus the optimal outcomes

may derive from the integration of multiple cues. In the stratified oral mucosa, the differential localization of IL-22RA1 and IL-17RA is important for this coordinated response. The implication of this pattern of receptor localization in other oral pathologies (such as oral mucositis and oral squamous carcinomas) and intrinsic relationships between the cells in these niches is yet to be fully appreciated. The role of IL-22RA1 in the BEL to support IL-17RA expression in the SEL bolsters the requirement for the integration of signals from these disparate receptors signaling pathways. At the transcriptional level, the coordinated induction of STATs, CEBPs, IκBζ, NFκB and AP1 transcription factors is likely to dictate optimal induction of key target genes and post-transcriptional modifications in OPC. While data is available for STAT3 and C/EBPs, the direct functional roles of IκBζ, NFκB and AP1 are yet to be demonstrated.

In this study, our observations were made by sublingual inoculation with a fixed dose of *C. albicans*. It is likely that altering the dose of the inoculum can influence the outcome of responses. The spectrum of epithelial-*Candida* responses is dependent on the density of hyphae. Invading hyphae cause epithelial damage which is part of the mechanism to discriminate between the transition from commensalism to virulence. Thus, the extent of damage dictates the ensuing host response. A previous *in vitro* study demonstrated that epithelial cells recognize *C. albicans* in a two-stage process which is determined by density and morphology of *C. albicans* [84]. The early response results in prolonged activation of NF-κB and a transient activation of MAPK signaling. At the later stage which corresponds to higher hyphal density, there is a second wave of prolonged MAPK signaling [84]. Thus, the threshold of hyphal burden can activate different pathways to lead to the induction of different genes and possibly trigger varied effector responses. While the concept of the threshold effect of invading hyphae on the oral mucosa has not been explored *in vivo*, it can potentially alter the outcomes of anti-*Candida* immune responses and may be worth exploring.

This can be done by infecting with different doses of *Candida* and followed by assessment of fungal burdens and thorough characterization of epithelial and immune responses.

6.3 Concluding Remarks

The findings from this dissertation have provided insights into the molecular mechanisms underlying IL-22/IL-22RA1-mediated signaling in acute and T cell recall OPC settings. The search for effective anti-fungal vaccine is still ongoing, with considerations for different immunogenic proteins and vaccination strategies. Vaccine efficacy is heavily dependent on the identification of immune correlates of protection. Our current data support previous findings in the field that suggest that in addition to IFN-γ and IL-17A meditated T cell responses, an effective oral antifungal vaccine must also elicit IL-22 responses [74, 107, 196, 230]. Since anti-IL-17A antibodies are used in the clinic for treatment of IL-17A-mediated proinflammatory conditions, particularly psoriasis [121, 224, 354, 355], our data suggest that co-administration of anti-IL-22 antibodies has potential to enhance susceptibility to fungal infections. Our T cell recall OPC data suggest the need for adoption of models that most closely reflect the observations in human OPC. While the acute OPC model has been instrumental in understanding and predicting the roles of innate IL-17RA responses in humans, recall infection models may provide further understanding, since OPC in humans is characterized by prolonged *C. albicans* antigen exposure. Thus, despite the advances made, some facets of oral antifungal immunity and oral mucosal immunity in general remain to be discovered.

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