THE ROLE OF IL-17 IN THE INNATE IMMUNE RESPONSE TO OROPHARYNGEAL CANDIDIASIS

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Oropharyngeal candidiasis (OPC; thrush) is an opportunistic oral infection caused by the commensal fungus *Candida albicans* that afflicts immunosuppressed and immunocompromised individuals. Although patients with HIV/AIDS are the prototypical population associated with development of thrush, there are a host of conditions that lead to susceptibility to OPC, including Hyper-IgE Syndrome (HIES). The autosomal dominant form of HIES (AD-HIES) is characterized by inherited dominant negative mutations in the STAT3 transcription factor, which is crucial for physiologic homeostasis and development in many tissues, including immune cells. Due to the ubiquitous nature of STAT3, mutations in this gene lead to a highly varied set of clinical sequelae, including oral thrush.

Numerous cytokines utilize STAT3 to mediate downstream signaling, including IL-6 and IL-23, which are crucial for the differentiation and expansion of T helper 17 (Th17) cells, respectively. IL-23 is necessary for 1) *in vivo* expansion of Th17 cells, and 2) the secretion of IL-17 and other proinflammatory cytokines. Recently, the Th17 lineage has been shown to play a major role in host defense against OPC. Our lab has previously shown that mice deficient in the IL-17 receptor (IL-17RA^{KO}) and IL-23p19 (IL-23^{KO}) are susceptible to OPC. Furthermore, saliva from both AD-HIES patients and IL-23^{KO} mice exhibits reduced ability to kill *C. albicans ex vivo*.

However, despite the requirement for both IL-17 and IL-23 in protecting against OPC, we found that CD4-specific STAT3 knockout (CD4(stat3^{KO})) mice are not susceptible to our model of OPC. These data suggested that STAT3 mediates the initial response to oral *C. albicans* challenge by inducing an IL-17-producing subset other than Th17. Experiments carried out in this study revealed that the initial source of IL-17 is lymphocytic, and is likely multifactorial, involving both γδ and αβ T cells. Furthermore, in this study, natural killer and

natural killer T cells were shown to have no apparent role in IL-17 secretion in response to *C. albicans* challenge in the oral cavity. In addition, while a memory or rechallenge response may involve CD4+ Th17 cells, this lineage appears to be unessential for the initial response to *C. albicans* in the oral cavity. Although we also attempted to examine the immune response to OPC using a mouse model of HIES harboring a transgenic STAT3 mutation, these mice proved resistant to *C. albicans* challenge at the buccal mucosa. However, despite the OPC resistance, they did exhibit a skin phenotype consistent with STAT3 deficiency, including eczematous lesions and delayed wound healing. These findings suggest that this mouse model may be used to study the role of STAT3 in both eczema as well as the immune response to cutaneous *Staphylococcus aureus* and *C. albicans* skin infections in the context of STAT3.

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PREFACE

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ABBREVIATIONS USED IN THIS DOCUMENT

Abbreviations used in this document are listed as follows in alphabetical order: Acquired Immunodeficiency Syndrome (AIDS), antimicrobial peptide (AMP), autosomal dominant HIES (AD-HIES), autosomal recessive HIES (AR-HIES), β-defensin (BD), canonical T helper 17 (Th17), chronic mucocutaneous candidiasis (CMC), granulocyte colony stimulating factor (G-CSF), Human Immunodeficiency Virus (HIV), Hyper IgE Syndrome (HIES), interleukin (IL),

Janus kinase (JAK), natural killer (NK), natural killer T cell (NKT), natural T helper 17 (nTh17), oropharyngeal candidiasis (OPC), pattern recognition receptor (PRR), retinoic-acid related orphan receptor (ROR), signal transducer and activator of transcription 3 (STAT3), T cytotoxic 17 (Tc17), transforming growth factor (TGF), wild type (WT)

1.0 INTRODUCTION

Oropharyngeal candidiasis is a common infection of immunocompromised populations, as well as persons at extremes of age. Although *C. albicans* is a human commensal and oropharyngeal candidiasis does not carry significant mortality, it does confer significant morbidity. Newborns, the elderly, and patients with cachexia (such as those with HIV/AIDS) are at significant risk of weight loss and dehydration due to odynophagia (pain with swallowing). Furthermore, patients who are susceptible to recurrent OPC require continuous antifungal prophylaxis or face regular disease exacerbations, leading to decreased quality of life. Study of the immune response to OPC, particularly the role of IL-17, may lead to novel therapeutic targets that help prevent the development of OPC and therefore decrease the morbidity of this disease.

1.1 THE TH17 LINEAGE AND THE ROLE OF STAT3 IN TH17 DIFFERENTIATION

The differentiation of CD4+ T helper cells into distinct functional lineages is an integral part of the adaptive immune response to specific pathogen challenges (1, 2). Differentiation into the Th1 lineage is associated with delayed-type hypersensitivity and immune responses to intracellular pathogens, while the Th2 lineage is associated with allergies/atopy and the response to helminthic challenge (1, 2). In recent years, a third effector T helper cell lineage, Th17, has been identified that redefines the original Th1/Th2 paradigm (1).

The differentiation of naïve CD4+ T cells to the Th17 lineage requires the cytokines IL-6, IL-21 and transforming growth factor β (TGF-β) (1, 3). IL-6 initiates the differentiation program via activation of Janus kinase 2 (JAK2), which phosphorylates tyrosine residues in the SH2

domain of STAT3. This triggers STAT3 dimerization and translocation to the nucleus to induce retinoic acid-related orphan receptor (RORγt) expression (Figure 1) (4). RORγt is the signature transcription factor for the Th17 lineage, and its expression is required for Th17 differentiation (1). IL-6 activation of Th17 differentiation occurs in concert with TGF-β, which acts to actively suppress the Th1 and Th2 differentiation pathways (3). Activation of STAT3 also induces IL-21 production and IL-21R expression, which acts in an autocrine fashion to upregulate RORγt and IL-17 expression (5). Subsequent expansion and potentiation of the Th17 lineage in vivo requires IL-23, which also signals through JAK2/STAT3. Activation of STAT3 by IL-23 induces production of the pro-inflammatory cytokines IL-17A and IL-17F (Figure 1) (1).

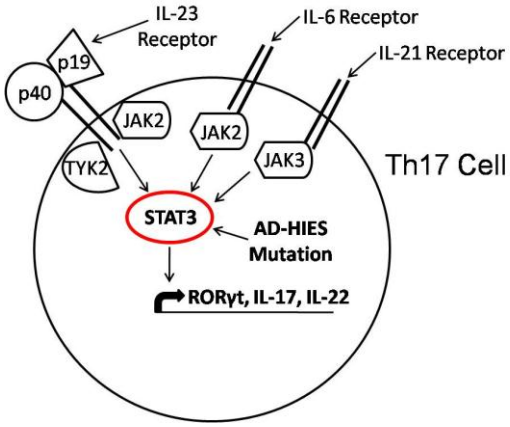


Figure 1: JAK/STAT3 signaling pathway mediates differentiation of Th17 cells

Th17 cells modulate the immune response by secreting several characteristic cytokines, including IL-17A, IL-17F and IL-22 (1, 6). Both IL-17A and IL-17F are members of the IL-17 family of cytokines, and bind the receptor IL-17RA. IL-17RA is ubiquitously expressed in tissues, suggesting that Th17 cells may play an extensive role in inflammation in both adaptive immunity and autoimmunity (1, 2, 7).

Several cell types other than canonical Th17 cells are known to produce IL-17, including γδ T cells, Natural Killer T (NKT) cells, some variants of natural killer (NK) cells and the newly described nTh17 subsets (8-12). γδ T cells are innate skin and mucosal resident T cells that express a limited TCR repertoire, and are integral to barrier immunity. γδ T cells are thought to

segregate similarly to αβ T cells in terms of cytokine production. $CD27^{-}CCR6^{+}$ γδ T cells have been shown to both express RORγt and produce IL-17, while $CD27^{+}$ γδ T cells produce IFNγ (12).

NKT cells are innate immune cells that recognize glycolipid antigens rather than peptides, and initiate the immune response to these molecules. Although NKT cells primarily produce IL-4 and IFNγ, a subset of these cells has recently been shown to secrete IL-17 as well as express RORγt and IL-23R. Traditional natural killer cells have also been reported to secrete IL-17, although the exact phenotype and role of IL-17-producing NK cells is still under study (12).

Recently, a variant of the canonical Th17 lineage known as natural Th17 (nTh17) cells was described by Tanaka, *et.al* and Marks, *et.al* (9, 11). nTh17 cells develop in the thymus and are partially dependent on IL-23/STAT3-mediated induction of IL-17 secretion. Once released into the periphery, these cells act as rapid responders at mucosal surfaces by mounting an inflammatory response without prepriming.

1.2 THE IMMUNE RESPONSE TO OROPHARYNGEAL CANDIDIASIS

1.2.1 A Brief Overview of *Candida albicans* Physiology and Pathogenesis

Candida is a large fungal genus encompassing over 150 species, only a handful of which cause disease in humans. The most common species in clinical isolates is *Candida albicans*, a commensal organism present in the oropharynx of up to 80% of healthy individuals (13, 14). Although *C. albicans* is a commensal, overgrowth of the fungi is responsible for a multitude of infections, including vulvovaginitis, systemic candidiasis, and oropharyngeal candidiasis (OPC) (15). These diseases rarely afflict healthy hosts, but immunocompromised persons are prone to recurrent severe infections by *C. albicans*. Recently, patients with hereditary primary immunodeficiencies that confer susceptibility to *Candida* have been described, but the vast majority of patients that develop *C. albicans* infections have acquired, or secondary, immunodeficiencies (16). Patients infected with Human Immunodeficiency Virus (HIV) who

subsequently develop Acquired Immunodeficiency Syndrome (AIDS) are particularly prone to OPC and esophageal candidiasis. In fact, over 90% of HIV/AIDS patients develop OPC at least once during the course of the disease, and many will have frequent recurrences (17). OPC is a common infection of immunocompromised persons, including infants and the elderly as well as patients with hematologic malignancies, who are post-transplant, or who have received head/neck radiation therapy (13, 18).

The most common manifestation of OPC is the formation of white, pseudomembranous plaques in the oropharynx, which is commonly asymptomatic. However, odynophagia and impaired taste sensation are common comorbidities, which can lead to weight loss and dehydration (13, 15). These symptoms are exacerbated by the development of esophageal candidiasis, a common complication of OPC in patients with HIV/AIDS (13, 15). There is no available vaccine for OPC, and thus it continues to be a major clinical problem.

1.2.2 The Role of Th17 Cells and IL-17 in the Immune Response to Oral Candidiasis

Prior to the identification of the Th17 lineage, the immune response to OPC was thought to be mediated primarily by Th1 cells. However, there is strong new evidence that IL-17 and Th17 cells are protective against *C. albicans*-mediated infections, including both OPC, mucocutaneous and disseminated candidiasis (19-21).

Th1 cells were believed to be the primary immune responder to OPC based in part on the observation that IL-12p40^{KO} mice are susceptible to OPC (22). Since IL-12 is a key cytokine in Th1 differentiation, susceptibility in these mice was attributed to loss of Th1 differentiation and thus Th1-mediated immunity (22). However, deficiency in IFN-γ, the primary Th1 cytokine, does not confer susceptibility to OPC, suggesting that Th1 cells may not be the key mediator of OPC resistance (22). The discovery of the Th17 lineage, and the role of IL-23 in its expansion, shed light on this paradox. IL-12 is composed of two subunits, p35 and p40. The p40 subunit is shared with IL-23, which is a heterodimer of the p40 and p19 subunits (23). Therefore, any data obtained using IL-12p40^{KO} mice must be interpreted in the context of both a Th1 and a Th17 deficiency.

To tease out the roles of Th1 versus Th17 deficiency in the host response to OPC, our laboratory infected IL-12p35^{KO} (IL-12^{KO}) and IL-23p19^{KO} (IL-23^{KO}) mice with *C. albicans* based on an infection model developed by the laboratory of Dr. Scott Filler (24). Briefly, mice were anesthetized and infected sublingually with a pre-weighed 2.5 mg cotton ball saturated in 2x10^7 organisms/mL of *C. albicans* strain CAF2-1 suspended in PBS. On Day 5 post-infection, one half of the tongue was harvested, homogenized, and then plated for assessment of fungal burden by colony enumeration (20).

As shown in Figure 2, both IL-12^{KO} and IL-23^{KO} mice show reduced clearance of the fungus at Day 5 post-infection compared to wild type (Figure 2A). However, the fungal burden in IL-12^{KO} mice did not result in overt OPC, whereas IL-23^{KO} mice show grossly visible pseudomembranous plaques, indicative of clinical disease (Figure 2B). In addition, the oral fungal burden in IL-23^{KO} mice was 2.6x10^4 per gram versus 1.4x10^3 in IL-12^{KO} mice (20). Furthermore, extension of the time course beyond Day 5 demonstrated that IL-12^{KO} mice were able to clear the infection by Day 17 (Figure 2C). This was in contrast to mice deficient in the IL-17RA subunit of the IL-17 receptor (IL-17RA^{KO}), which are not responsive to IL-17, and who maintained a constant fungal burden throughout the 17 day experiment (20). This is consistent with the fact that IFNγ mice are resistant to OPC (22). Taken together, these data demonstrate that the IL-23/IL-17 axis of immunity is crucial in OPC resistance, whereas Th1 cells are less important.

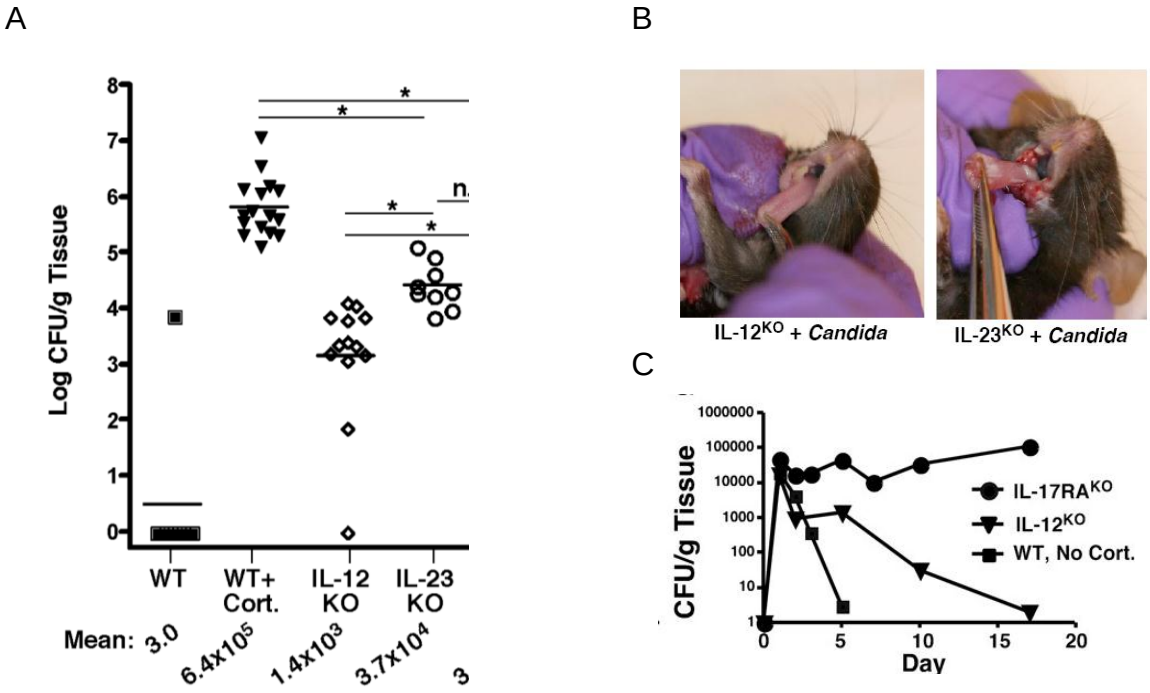


Figure 2: IL-23^{KO} mice show increased susceptibility to OPC compared to IL-12^{KO} mice.
Experiments performed by Dr. Heather R. Conti (20). *, p <0.05 (Mann-Whitney test).

Several other groups in have demonstrated a role for IL-17 in resistance to both OPC and other infections caused by *C. albicans*. Activation of specific fungal pattern recognition receptors (PRRs) on monocytes and dendritic cells, such as the mannose receptor, dectins 1 and 2, and the NLRP3 inflammasome, induce production of IL-1β, IL-6, and IL-23 leading to Th17 differentiation (Figure 3) (25-28). Furthermore, differentiated Th17 cells express the chemokine receptor CCR6, which mediates migration to the skin and mucosal tissues, supporting a role for these cells in oral fungal infections, including OPC (29). In addition, secretion of IL-17A/F and IL-22 by Th17 cells induces production of antimicrobial peptides (AMPs) such as beta-defensin 2 as well as granulocyte colony-stimulating factor (G-CSF) and CXC chemokines at mucosal epithelial surfaces (30, 31). Chemokines in the oral cavity act to recruit neutrophils to the oral mucosa, which is further amplified by G-CSF-mediated granulopoeisis (7, 26). Taken together, these data strongly suggest a role for IL-17 and Th17 cells in mediating resistance to OPC by

inducing the recruitment of neutrophils and the expression of antimicrobial peptides in the oral cavity. The putative mechanism by which this occurs is outlined in Figure 3.

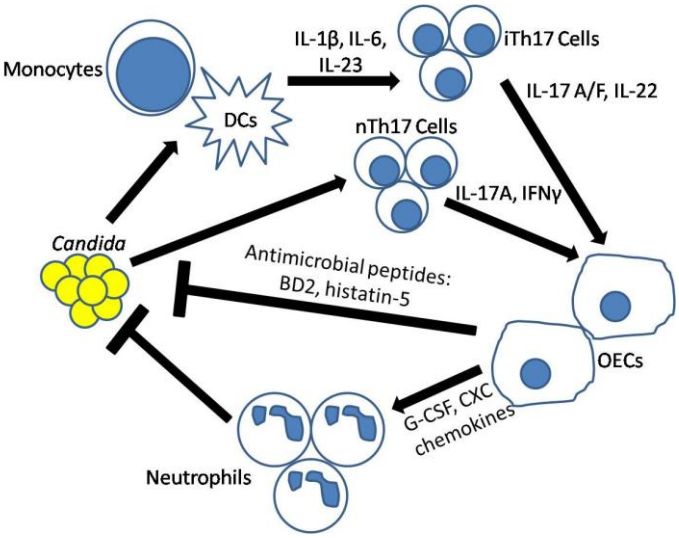


Figure 3: Putative immune response to oral challenge with *Candida albicans*

1.3 PRIMARY IMMUNODEFICIENCIES AND OPC: HYPER-IGE SYNDROME

Several primary immunodeficiencies in humans have been shown to confer susceptibility to *C. albicans* infections. Characterizing the mutations responsible for these immunodeficiencies have supported the laboratory findings that both PRRs and IL-17 are crucial for resistance to this organism. These primary immunodeficiencies can be loosely grouped into two categories: those involved in IL-17 and Th17 signaling, and those involved in recognition of *C. albicans*.

Recently the first cases of primary IL-17 cytokine and IL-17 receptor deficiencies were reported by Puel, *et al*. (16). Patients with mutations in either IL-17F or IL-17RA were described to suffer from severe chronic mucocutaneous candidiasis (CMC). CMC presents clinically with recurrent *C. albicans* infections at mucosal surfaces, including the oral and genital tracts, and the skin.

However, these direct mutations in IL-17 and its receptor are extremely rare. Rather, mutations in the gene encoding STAT3 are the most common form of IL-17 and Th17 deficiency. Patients with STAT3 mutations present with autosomal-dominant Hyper IgE Syndrome (AD-HIES), a disorder of variable penetrance that is associated with a constellation of clinical features. AD-HIES is characterized by mucocutaneous candidiasis (including recurrent OPC), severe eczema, recurrent *Staphylococcus aureus* infections, high serum IgE levels and characteristic facial and skeletal abnormalities. Although AD-HIES patients suffer from recurrent OPC, disseminated candidiasis and other fungal infections are rare in these patients (32, 33). In the majority of patients with AD-HIES, mutations have been localized to the gene encoding STAT3. Dominant negative mutations in the DNA binding or SH2 domains of STAT3 lead to a greater than 50% reduction in STAT3 activity. This reduction leads to both Th17 and IL-17 deficiencies due to defects in IL-6 and IL-23 signaling, and ultimately causes susceptibility to diseases such as recurrent OPC (34-36).

There is an autosomal recessive form of HIES (AR-HIES), which exhibits a very similar syndrome to that of the autosomal dominant form. However, AR-HIES patients also exhibit increased susceptibility to cutaneous viral infections, which is not found in AD-HIES. The hereditary mutations responsible for AR-HIES have not yet been characterized, although one patient with a documented Tyk2 (Janus kinase family member that phosphorylates STAT3) mutation who presented with an AR-HIES-like syndrome has been reported (37). Despite this finding, further genetic analysis of other AR-HIES patients did not reveal any other cases of Tyk2 mutation (38).

Although there are several known mutations in the PRRs and recognition pathways involved in resistance to *Candida albicans*, detailed descriptions of these disorders are beyond the scope of this thesis. In brief, human patients with mutations in the PRR Dectin-1 and the adaptor molecule CARD-9 both demonstrate recurrent mucocutaneous candidiasis secondary to reduced IL-17 responses to fungal challenge (21, 39).

Taken together, these striking findings in humans recapitulate much of the putative role of IL-17 and Th17 in OPC generated in basic science laboratories. These results provide exciting new treatment avenues to pursue within the IL-17 and the Th17 pathway, which could have significant implications considering the numerous pathologies in which IL-17 is implicated, including Rheumatoid Arthritis, Multiple Sclerosis and Inflammatory Bowel Disease.

1.4 SALIVARY CONTRIBUTION TO ORAL MUCOSAL IMMUNITY

Saliva plays a major role in resisting oral infections, serving as a mechanical clearance mechanism and as a source of AMPs (40). The vital role of saliva is dramatically illustrated by OPC susceptibility in the context of clinical conditions: 1) Patients who have undergone head/neck irradiation have relatively normal adaptive immune systems, but are highly susceptible to OPC and 2) Sjögren's syndrome patients also experience frequent bouts of OPC due to decreased *C. albicans* clearance because of severely reduced salivary flow (17, 41, 42).

Whole saliva is derived from the buccal mucosa, gingival crevices and salivary glands, and is instrumental in oral barrier immunity (40). Salivary flow and AMPs such as histatins, defensins and calprotectins (S100A8/9) inhibit overgrowth and epithelial invasion of *C. albicans* (6, 43). Upon contact with *C. albicans*, oral epithelial cells and oral keratinocytes upregulate secretion of fungicidal factors, including the AMPs beta-defensin 2 and 3 (BD2, 3) (43). This secretion is thought to be enhanced by IL-17A and IL-22, both of which are produced by Th17 cells (6). Data recently published by our group indicates both IL-17 and IL-23 help to control the anti-fungal activity of saliva, and therefore reveal an unexpected role for these cytokines in protecting against OPC. Our lab has previously shown by microarray that tongues harvested post-infection from IL-17RA^{KO} mice show decreased expression of S100A8, S100A9 and BD3 compared to WT (20).

Concordantly, the laboratory previously published found that the candidacidal activity of saliva from both IL-23^{KO} and IL-17RA^{KO} mice is considerably reduced compared to saliva from WT mice. Furthermore, this finding was correlated in human patients with HIES syndrome, whose saliva also showed decreased ability to kill *C. albicans* when compared to healthy controls. (Figure 4) (20, 44). Candidacidal activity was performed as previously described (20, 44). Briefly, salivary secretion was induced by carbachol injection and saliva collected for 10 minutes. Saliva was then incubated with *C. albicans* for two hours and plated on YPD for colony enumeration. Saliva from IL-23^{KO} mice exhibited reduced candidacidal activity compared to both WT and IL-12^{KO} mice on both Day 0 and Day 1, whereas saliva from IL-17RA^{KO} mice showed reduced candidacidal activity only on Day 1 (Figure 4A). In agreement with the mouse data, AD-HIES patient saliva exhibited significantly reduced candidacidal activity compared to normal controls (Figure 4B).

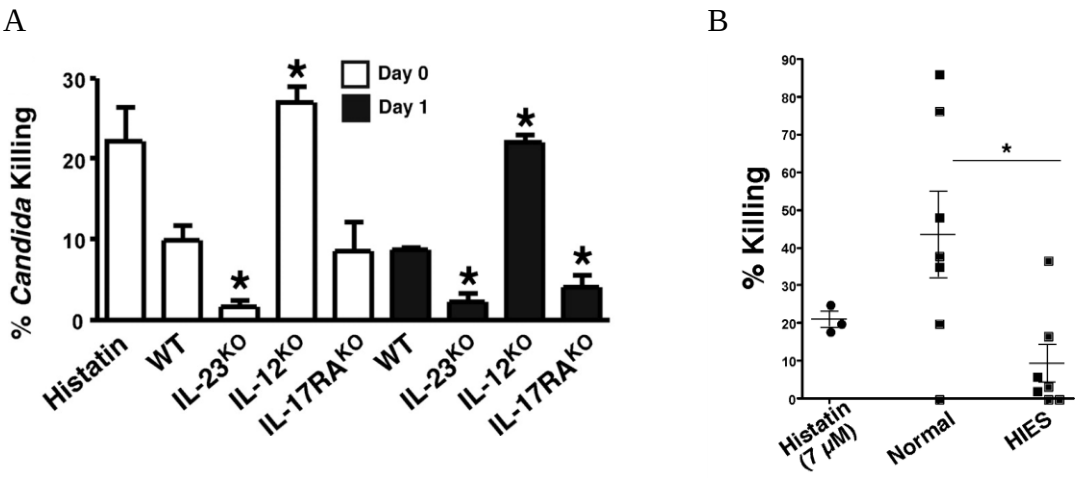


Figure 4: Saliva from IL-23^{KO} and IL-17RA^{KO} mice, and AD-HIES patients shows reduced candidacidal activity compared to healthy controls. Experiments performed by Dr. Heather R. Conti (20).*, p <0.05 (unpaired Student's *t*-test).

Since IL-23 is required for expansion of the Th17 cell population and for secretion of IL-17 by both Th17 and innate immune cells, these data suggest that impaired function of Th17 cells and the interplay between IL-23 and IL-17 reduces the fungicidal activity of saliva, which may negatively impact host resistance to OPC. However, the mechanisms by which IL-17 and IL-23 mediate salivary OPC resistance are still yet to be elucidated.

2.0 RESEARCH DESIGN AND METHODS

2.1 MICE

Wild type (WT) C57BL/6, WT B6129SF2/J, TCRγδ^{KO}, TCRαβ^{KO}, Prkdc^{scid}, CD4^{KO}, CD8^{KO}, and SCIDγc^{KO} mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Floxed CD4+ T cell specific conditional STAT3^{KO} (CD4(stat3^{KO})) and STAT3-transgenic (HIES) mice were provided as a generous gift from the laboratory of Dr. J.J O'Shea at the NIH (Bethesda, MD). IL-23p19^{KO} mice were bred in-house, but had been previously provided by the laboratory of Dr. Jay K. Kolls at LSU (New Orleans, LA). Unless otherwise specified, all mice used were on the C57BL/6 background, 6-10 weeks of age and exclusively female or male to minimize variability due to salivary gland differences between sexes (45). Cohorts of nine mice have sufficient power to achieve statistical significance of $p<0.05$ (20, 24). All mice were housed in the Thomas E. Starzl South Biomedical Sciences Tower at the University of Pittsburgh. Mice were housed initially in a specific pathogen free corridor in microisolator caging and given only food, water and bedding that had been autoclaved. For infection, mice were infected in a BSL-2 corridor in the same building to protect both animals and personnel, which also adhered to SPF protocols and used only autoclaved supplies.

2.2 ORAL INFECTION WITH CANDIDA ALBICANS

Infection with *Candida albicans* strain CAF2-1 and collection of saliva was performed as published by Kamai *et. al.* (24) and our lab (20). Briefly, mice were infected sublingually with a 2.5 mg cotton ball saturated in 2x10^7 organisms/mL of *Candida albicans* strain CAF2-1

suspended in PBS. On Days 4 or 5 post-infection, mice were sacrificed and the tongue and kidney harvested for analysis. Susceptibility to OPC was determined by tracking weight loss over the course of infection followed by tongue homogenization to determine total oral fungal burden. For humane purposes and to minimize suffering, mice were euthanized when weight loss reached greater than 30% of initial body weight. Gross and histologic examination of the tongue for pseudomembranous plaques and the presence of *Candida* was performed by Hematoxylin and Eosin and Periodic Acid Schiff staining, respectively (20). Data were compared to untreated, uninfected WT (sham), untreated, infected WT (negative control) and infected, cortisone-treated WT mice (positive control). Our experimental protocol for establishing OPC infection has demonstrated that development of overt disease occurs in WT mice after immunosuppression with administration of 225 mg/kg cortisone acetate on Days -1 before infection and Days 1 and 3 post-infection. Disease also develops in both IL-17RA^{KO} and IL-23^{KO} mice without additional immunosuppression demonstrating the role of Th17 cells in this model (20). The kidney was also homogenized in each mouse to ascertain if disseminated candidiasis (candidemia) had occurred secondary to oral challenge. To determine if statistically significant differences existed between the oral fungal burdens of different cohorts, an unpaired Student's *t*-test with Welch's correction was performed, unless otherwise specified in the figure legend.

2.3 COLLECTION AND ANALYSIS OF MURINE SALIVA

Baseline salivary secretion in mice is insufficient to perform candidacidal testing. Therefore, salivary secretion was induced by intraperitoneal injection of 10ul of 100ug/ml carbamoylcholine chloride in phosphate-buffered saline (Sigma-Aldrich, St. Louis, MO). Saliva was then collected directly from the oral cavity using a micropipette. Collected saliva was centrifuged for five minutes at 2500 rpm and supernatant removed and placed in another tube, discarding the pelleted proteins. The supernatant was then incubated at a 3:1 ratio with 10^4 organisms of *C. albicans* for two hours and plated in triplicate on YPD agar for colony enumeration. Histatin-5 (a histidine-rich antifungal peptide found in human saliva purchased

from AnaSpec, San Jose, CA) was used as a positive control and *C. albicans* alone was used as a negative control.

Oral amylase activity in murine saliva was assessed using the EnzChek *Ultra* Amylase Assay Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol (46). However, human salivary α-amylase (Sigma-Aldrich) was used to create the standard curve to measure murine salivary α-amylase concentrations in lieu of the α-amylase derived from *Bacillus* spp. that was recommended by the kit. Fluorescent activity in each sample was determined by kinetic assay using FluroNunc™ 96-well flat bottom black plates (Nunc, Roskilde, Denmark) during which fluorescence was measured in five minute intervals using the Synergy 2 Microplate Reader (BioTek, Winooski, VT).

Oral chitinase activity was detected using the Fluorometric Chitinase Assay Kit (Sigma-Aldrich) according to the manufacturer's protocol. All data shown depicts the concentration of chitinase in each sample based on cleavage of the 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide substrate provided by the kit, which measures β-N-acetylglucosaminidase activity. As in the amylase assay, activity was measured using a kinetic assay on the Synergy 2 microplate reader on FluroNunc™ 96-well black plates. The concentration of chitinase in murine samples was calculated using the formula detailed in the manufacturer's protocol based on the results from internal standards provided by the kit.

For both α-amylase and chitinase, the fluorescent signal from each sample at each time point was first normalized by subtracting the appropriate substrate control (no enzyme) to eliminate background fluorescence. Once the normalized value was obtained, this was used to plot the activity of the samples over time (shown in Figure 9A and B).

2.4 GENERATION OF STAT3-TRANSGENIC (HIES) MICE

Homozygous somatic mutations or deletions in STAT3 are embryonic lethal; therefore the most common models of STAT3 deficiency utilize a Cre-Lox system to study the effect of loss of this transcription factor on specific tissues (47, 48). Varied Cre-mediated tissue-specific STAT3 deletion studies have shown that adult mice with STAT3 defects exhibit many abnormalities

such as delayed wound healing, thymic hypoplasia and hematopoietic defects, depending on the affected tissue (48). Although this approach is useful for studying the effects of STAT3 on a single tissue, these mouse models do not accurately represent HIES patients, who have a heterozygous STAT3 mutation that is expressed on a whole body level. Therefore, to create a mouse model of HIES, a tissue-nonspecific transgenic approach is appropriate.

The HIES mouse model (STAT3-tg) was generated at the laboratory of Dr. John J. O'Shea at NIAID, NIH. In brief, HIES mice were generated by inserting a STAT3 transgene containing a deletion of Valine 463 as well as partial deletion of the adjacent intron (Figure 9). From the transgenic embryos selected, the line studied in this paper harbors five copies of the mutant transgene on the X chromosome. This mouse is thought to be similar to AD-HIES patients in the sense that V463 is considered a "hot spot" mutation site in HIES patients that reduces STAT3 activity by greater than 50% (49). Although the HIES mice express five copies of the mutant transgene on chromosome X, the endogenous STAT3 genes remain unaffected. Therefore the mouse is able to generate sufficient wild-type STAT3 homodimers to survive to adulthood, but is predicted to demonstrate adult tissue defects and susceptibility to infection similar to HIES patients due to the risk of heterodimer or mutant homodimer generation using the mutant STAT3 gene product.

2.5 T HELPER CELL DIFFERENTIATION AND FLOW CYTOMETRY

T helper cells were differentiated from CD4+ splenocytes as follows. Whole spleens were sterilely harvested and placed immediately into CRPMI (Gibco, Carlsbad, CA) media on ice. A single cell suspension was generated by dissociating the organ and passing the homogenized tissue through a 40 micron cell strainer. Red cell lysis was carried out using 2 ml of sterile Gey's solution, incubating the cells for four minutes at room temperature, and then neutralizing the reaction with an excess of CRPMI. Cells were then washed once using sterile 1X PBS.

To isolate CD4+ T cells, negative selection was performed by labeling the cells using the CD4+ T Cell Isolation Kit and collecting the CD4+ fraction on using an autoMACS according to the manufacturer's protocol (Miltenyi-Biotec, Bergisch Gladbach, Germany). An

aliquot of CD4+ cells was taken and resuspended in CRPMI containing only 27.5 uM β-mercaptoethanol (Gibco) to be used for unstimulated controls. The remaining CD4+ cells were resuspended in CRPMI supplemented with 2 ug/ml anti-CD3 antibody (BD Pharmingen), 2 ug/ml anti-CD28 antibody (eBiosciences, San Diego, CA), 20 U/ml rmIL-2 (R&D Systems, Minneapolis, MN) and 27.5 uM β-mercaptoethanol. Cells were plated in 24-well culture dish at a density of 1M cells per well and the following antibodies and differentiating cytokines were added. For Th0 cells (used as a staining control), no additional reagents were used. For Th1 differentiation, 10 ug/ml anti-IL-4 (eBioscience) and 10 ng/ml IL-12 (R&D Systems) was added. For Th17 differentiation, 30 ng/ml IL-6 (R&D Systems), 50 ng/ml IL-23 (R&D Systems), 5 ng/ml hTGF-beta1 (R&D Systems), 10 ug/ml anti-IL-4 and 10 ug/ml anti-IFN-γ (eBioscience) was added.

On culture Day 3, an additional milliliter of CRPMI supplemented with 27.5 uM β-mercaptoethanol and 20 U/ml rmIL-2 was added to each well. Cells were then harvested on Day 6 and the percentages of Th1 and Th17 cells determined by flow cytometry. Fluorescent cytokine staining for IL-17 and IFNγ was carried out by both traditional intracellular staining and by surface staining using the IFNγ and IL-17 Secretion Assays according to the manufacturer's protocol (Miltenyi-Biotec).

Intracellular staining was performed as follows. Cells were stimulated at 37°C for 5-8 hours using 1.5 ug/ml ionomycin (Sigma-Aldrich), 50 ng/ml PMA (Sigma-Aldrich) and 1 ul GolgiPlug (BD Biosciences) per 1x10^6 cells suspended in 1 ml CRPMI. After stimulation, cells were kept on ice all the time unless indicated and stained at a concentration of 1x10^6 cells per sample. An initial Fc blocking step was performed to minimize non-specific binding by incubating each sample in 100 µl of FACS Buffer supplemented with 0.5ug of Fc block (CD16/32, eBioscience) and incubated on ice for 10 minutes. Surface staining for CD4 was then performed prior to intracellular staining by resuspending the cells in 100µl of FACS buffer containing CD4-FITC (BD Pharmingen). Single color controls for IL-17 and IFN-γ were surface stains using MHC Class II-PE (eBioscience) and CD3-APC (BD Pharmingen) respectively. Cells were incubated with surface antibodies for 30' on ice in the dark.

After completion of surface staining, cells were fixed using the BD Cytofix/Cytoperm Fixation/Permeabilization Kit according to the manufacturer's instructions. Cells were then

stained with IFNγ-APC (BD Pharmingen) and/or IL-17-PE (BD Pharmingen) and incubated for 30’ on ice and in the dark.

For surface IL-17 and IFNγ staining, the appropriate secretion assay was used according to the manufacturer’s instructions. In brief, cells were stimulated at 37°C for 3 hours using 1 ug/ml ionomycin and 10 ng/ml PMA per 1x10^6 cells suspended in AIM V media (Invitrogen) supplemented with 27.5 uM β-mercaptoethanol. Cells were then labeled with both IL-17 and IFNγ catch reagents and cytokines allowed to secrete for 45 minutes with shaking at 37°C. Cells were then stained with biotinlyated IL-17, followed by surface staining for IFN and biotin according to the manufacturer’s instructions. Cells were also stained for CD4 as described above.

Samples were acquired on an LSR II (BD Biosciences) and all data analyzed using FlowJo version 7.6.4 (Tree Star, Inc., Ashland, OR).

3.0 RESULTS

3.1 IL-17 PRODUCTION IN RESPONSE TO ORAL CHALLENGE BY CANDIDA ALBICANS IS MULTIFACTORIAL, BUT CRUCIAL FOR IMMUNE RESISTANCE

3.1.1 Although CD4-specific STAT3 Deficient Mice Are Not Susceptible to OPC, RORγt^{KO} Mice Develop Disease

Due to the fact that HIES patients are known to have both Th17 and IL-17 deficiencies, which are thought to contribute to these patients' recurrent thrush, we first assessed OPC susceptibility in a CD4(stat3^{KO}) mouse to confirm the crucial role of Th17 cells in resisting OPC. Since STAT3 is required for Th17 differentiation, and all T helper cells express CD4, we expected that CD4(stat3^{KO}) mice, which do not produce any Th17 cells, would show increased susceptibility to OPC due to this defect (34). Despite previous data published by our laboratory that suggested the canonical Th17 lineage mediated the IL-23/IL-17 immune response to OPC, and thus conferred resistance (20), CD4(stat3^{KO}) mice were completely resistant to infection (Figure 5). Both CD4(stat3^{KO}) mice (STAT3 FC), as well as stat3$^{fl/fl}$Cre^{neg} (STAT3 FW) completely cleared the infection by Day 5, similar to wild type C57BL/6 mice (Figure 5A). To minimize the possibility that this finding was due to experimental error, virulence of the organism was assured by the high fungal burden of cortisone-treated WT mice (positive control), and the development of OPC by IL-23^{KO} mice. We further confirmed that the CD4(stat3^{KO}) mice we infected demonstrated a Th17 differentiation defect by inducing differentiation of whole splenocytes (Figure 5B).

In light of the result that STAT3 deficiency in the CD4 compartment did not confer susceptibility to OPC, we examined the role RORγt using a knockout mouse model. RORγt

expression is necessary for production of IL-17 in both T cells and innate IL-17-producing cell types. Although loss of a single allele of RORγt did not affect the mouse's ability to resist oral *C. albicans* challenge, the homozygous knockout mouse was found to be more susceptible than IL-23^{KO} mice, suggesting a major role for RORγt-mediated IL-17 expression in the immune response to OPC (Figure 5C).

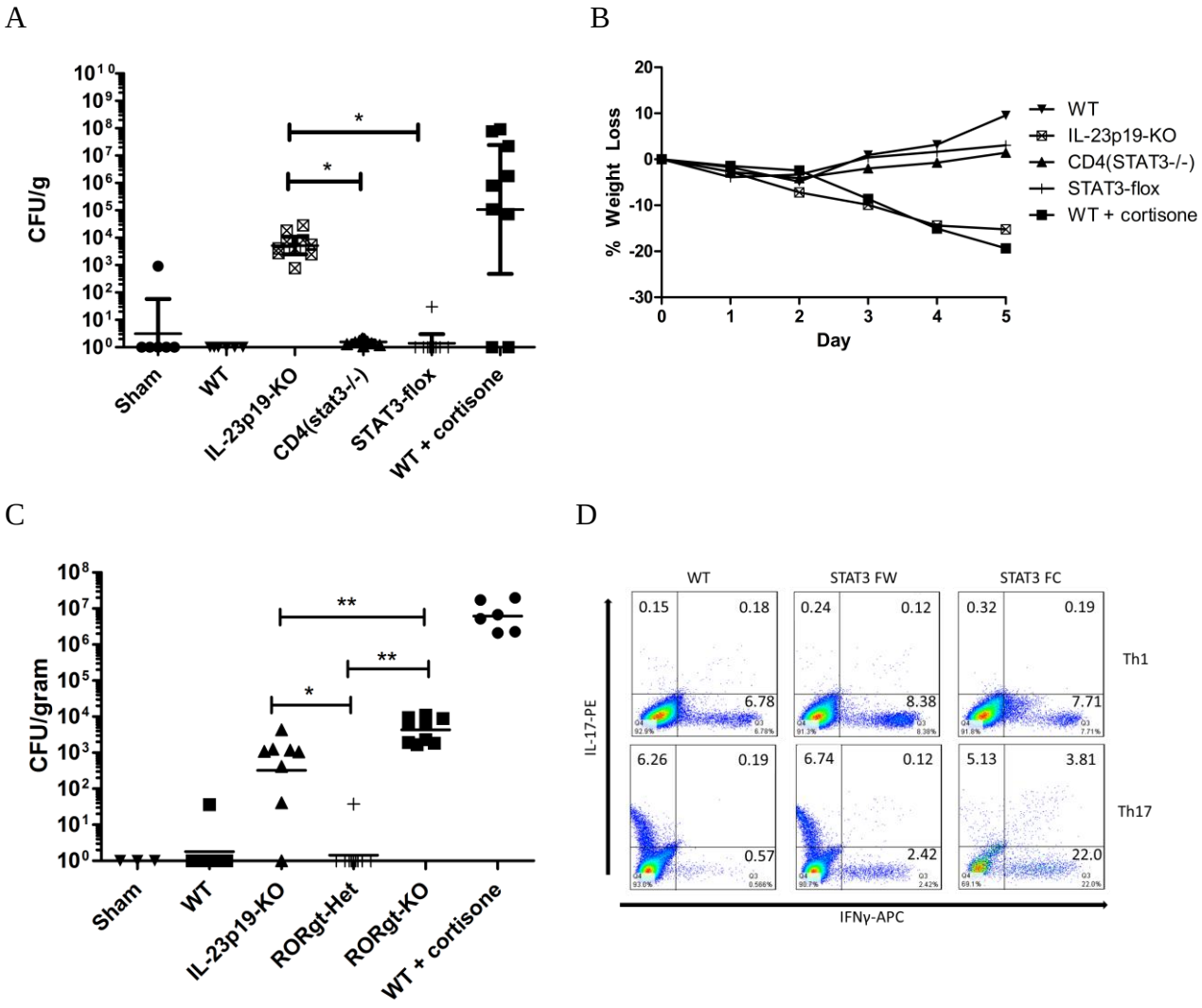


Figure 5: Although CD4(stat3^{KO}) mice resist OPC, RORγt^{KO} mice are strikingly susceptible.
A and C – Results of OPC challenge of CD4(stat3^{KO}) and of RORγt^{KO} mice; STAT3 FW=STAT3$^{fl/fl}$, Cre^{neg}. *, p = <0.05, **, p = <0.01. Significance determined by unpaired student's *t*-test with Welch's correction. **B –** Percentage weight change compared to Day 0 over the course of the five day infection. **D –** Results of T helper cell differentiation of whole splenocytes. Total cell viability was decreased in Th17 differentiated STAT3 FC CD4+ splenocytes, thus although IL-17+ cells were found at frequency of 5.13%, this did not represent a comparable absolute number of IL-17 producing cells as WT or STAT3 FW. Data representative of two experiments. N=5 for each cohort except sham and WT, where N=3.

3.1.2 Sole Deficiency in CD4 or CD8 T cells Is Not Sufficient to Confer Susceptibility to OPC, but Loss of the Entire Lymphocyte Lineage Causes Profound OPC

Although IL-23^{KO} mice develop evidence of overt thrush and IL-12^{KO} mice do not, the latter mice do harbor a significant oral fungal burden on Day 5, which is eventually cleared by Day 17 (Figure 2). These data suggest that although IL-17 is crucial for resistance of thrush, there may still be a role for Th1 cells in resistance of OPC. To ascertain whether there may have been a compensatory response of Th1 cells in the CD4(stat3^{KO}) mice that allowed these mice to resist OPC, we tested the susceptibility of CD4^{KO} mice, which are deficient in all T helper lymphocytes compared to CD8^{KO} mice, which are deficient in all T cytotoxic lymphocytes. Unexpectedly, both CD4^{KO} and CD8^{KO} mice regained all lost weight and cleared the *C. albicans* challenge by Day 5 (Figure 6A and B). These findings, in conjunction with the fact that CD4(stat3^{KO}) mice are resistant to the development of OPC, suggest that the major contribution to the early response (less than five days) to oral challenge with *C. albicans* is due to an innate cell type that does not express either CD4 or CD8. However, it is certainly possible that compensatory mechanisms in CD4^{KO} and CD8^{KO} mice are responsible for the initial resistance to OPC, and that these cell types still comprise a significant part of the immune response.

Upon determining that neither CD4^{KO} nor CD8^{KO} were susceptible to OPC, we infected IL-2Rγc^{KO} mice, which are deficient in all cytokines that rely on the common γ chain (IL-2, IL-4, IL-7, IL-9 and IL-15). These mice are therefore profoundly immunosuppressed and exhibit defects in both production and function of T lymphocytes (including $\gamma\delta$ T cells), myeloid cells, natural killer cells, and B lymphocytes, as well as lymphoid tissue structural defects. Although the effects of deficiency in the common γ chain are broad and affect nearly all areas of the immune system, studying the IL-2Rγc^{KO} mice allowed us to begin to delineate which cell type is the primary IL-17 producer in the initial response to oral challenge with *C. albicans* by isolating which cytokines were responsible for maintenance of the cell type. As expected, infection of IL-2Rγc^{KO} with oral candidiasis led to profound weight loss, surpassing that of cortisone-treated wild type mice, and an inability of the mice to survive beyond Day 4 post-infection (Figure 6C and D).

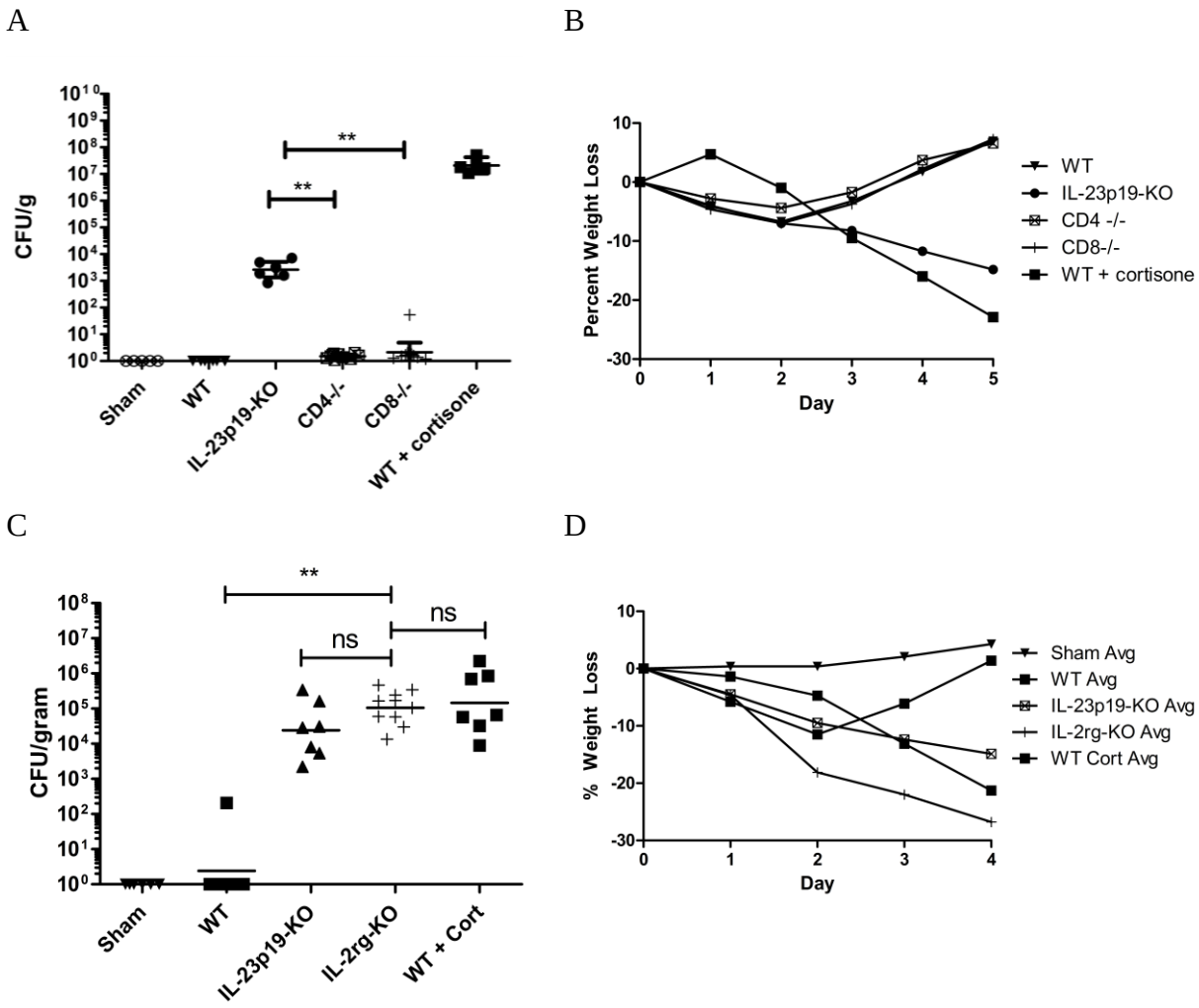


Figure 6: IL-2Rγc^{KO} mice succumb to oral infection with *C. albicans* while mice deficient in solely CD4 or CD8 are able to completely resist infection. A and C – Colony forming units/gram of tongue on Day 5. **, p = <0.01. Significance determined by unpaired student's *t*-test with Welch's correction. **B and D –** Percentage weight change compared to Day 0 over the course of the five day infection.

3.1.3 Deficiency in Natural Killer and Natural Killer T Cells Does Not Affect the Immune Response to Oral *C. albicans* Challenge

The finding that IL-2Rγc^{KO} mice are profoundly susceptible to OPC while CD4^{KO} and CD8^{KO} are not suggested the possibility that the early innate immune response to oral *C. albicans* challenge is not CD4 or CD8 T lymphocyte mediated, but rather primarily mediated by an innate IL-17-secreting cell such as NK, NKT or γδ T cells. To determine the potential role for NK and NKT cells, we took a two-pronged approach that examined the susceptibility of IL-15Rα^{KO} mice as well as that of Prkdc^{scid} (SCID) mice. IL-15 is essential for the development of both NK and NKT cells, as well as preventing apoptosis of lymphocytes. Therefore, IL-15Rα^{KO} mice have abnormal NK cell production as well as a complete absence of NK cytolysis and a significant decrease in NKT cells (50, 51). Furthermore, these mice exhibit defects in the CD8 T cell and intraepithelial γδ T cell compartments (52). On the other hand, SCID mice harbor a mutation in *Prkdc*, a protein kinase gene, which leads to severely decreased B and T cell numbers, but an increase in NK cell number and cytolytic activity, as well as an increase in macrophage, monocyte and granulocyte numbers (53).

Upon oral challenge with *C. albicans*, IL-15Rα^{KO} mice were found capable of completely clearing the fungus whereas the SCID mice developed overt thrush over the course of the five day infection (Figure 7). Since the IL-15Rα^{KO} mice were able to clear the pathogen despite the fact that they lack NK cell cytolytic activity while SCID mice, which have hyperactive NK cells, were susceptible to OPC, we can conclude that NK cells do not play a significant role in the immune response to oral challenge with *C. albicans.*

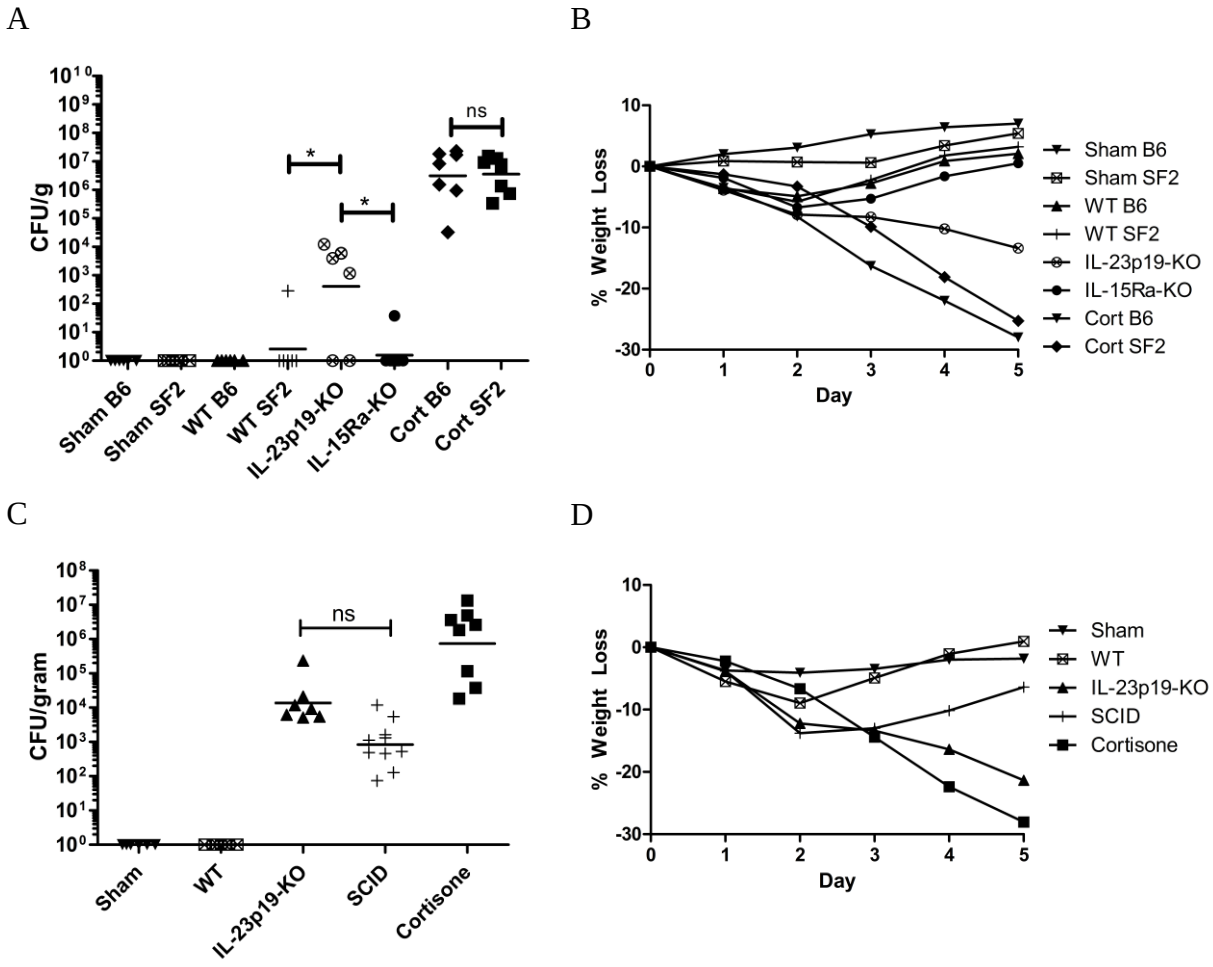


Figure 7: IL-15^{KO} mice completely resist OPC despite a complete lack of NK cells, while SCID mice develop overt disease with hyperactive NK cells. A and C – Colony forming units/gram of tongue on Day 5. B6 = C57BL/6 WT mice; SF2 = B6.129SF2/J hybrid WT mice. *, p = <0.01. Significance determined by unpaired student's *t*-test with Welch's correction. **B and D –** Percentage weight change compared to Day 0 over the course of the five day infection.

3.1.4 The γδ T Cell Compartment is Not the Primary Source of IL-17 in the Immune Response to Oral Challenge with *C. albicans*

The confirmation that the major source of IL-17 in response to *C. albicans* in the oral cavity is not NK or NKT cells reaffirmed the hypothesis that the primary mediator of IL-17 secretion is a member of the T lymphocytic compartment. However data CD4 and CD8 knockout mice cast significant doubt that acquired T cell immunity plays a major role in the initial immune response, suggesting a role for buccal mucosal-resident γδ T cells. Previous data published by our lab demonstrated that TCRγδ^{KO} mice are only partially susceptible to OPC, exhibiting low oral fungal burdens and no evidence of overt disease (20). We reaffirmed these findings by challenging TCRγδ^{KO} mice side-by-side with TCRαβ^{KO} mice (Figure 8). As shown in the Figure 8A, the TCRγδ^{KO} mice were only partially susceptible, exhibiting a bimodal distribution of either no fungal burden, or a low fungal burden, which is very similar to our published data (20). In contrast the TCRαβ^{KO} mice completely cleared the pathogen, similar to WT. Although the TCRγδ^{KO} mice showed variable susceptibility, in conjunction with published results, these data suggest that deficiency in γδ T cells is insufficient to confer susceptibility to thrush in the absence of other immunodeficiency.

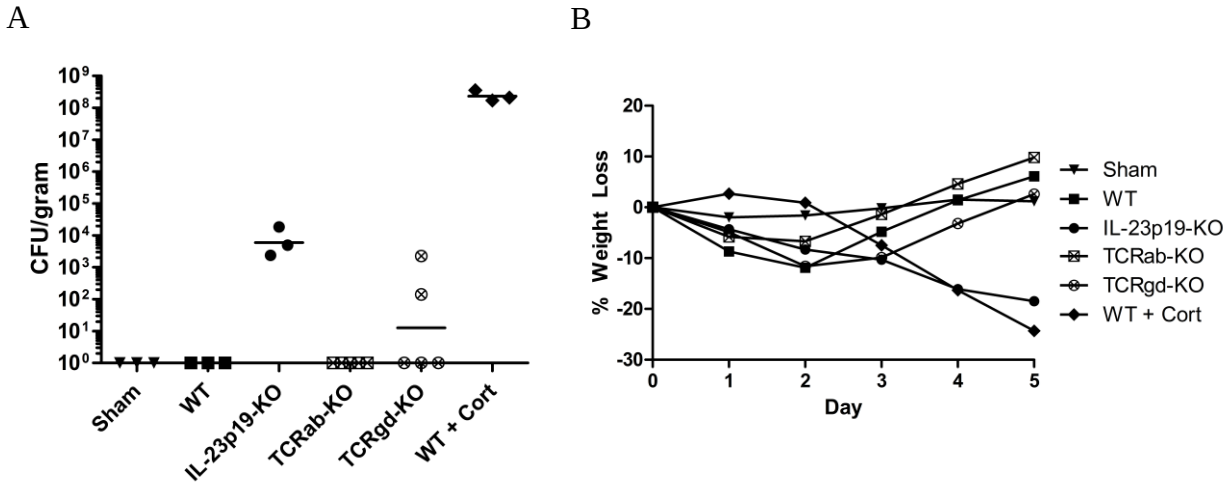


Figure 8: TCRγδ^{KO} mice are only partially susceptible to OPC while TCRαβ^{KO} mice are resistant. **A –** Colony forming units/gram of tongue on Day 5. **B –** Percentage weight change compared to Day 0 over the course of the five day infection.

For ease of reference, Table 1 details the susceptibility profile of the knockout strains tested in our OPC model (Figures 5, 6, 7, and 8), as well as a brief summary of the immune defects found in these mice. Taken together, the knockout mouse susceptibility data strongly suggests that NK and NKT cells play little or no role in the initial immune response to OPC. Furthermore, data in all of the different iterations of T cell knockout mice suggests that both the γδ and αβ T cell lineages may be involved in resisting oral challenge with OPC, but that the extent of this may be masked in any particular mouse model due to compensatory responses.

Table 1: Summary Table of OPC Susceptibility in Mouse Models of Immunodeficiency

Knockout Mouse	Susceptible?	Immunodeficiency (with respect to Th17/IL-17)
IL-23p19	Yes	• Impaired maintenance of Th17 cells *in vivo* • Decreased IL-17 production
IL-17RA	Yes	• Response to IL-17A and IL-17F absent in all tissues
IL-12p35	±	• Th1 cells absent
CD4(stat3)	No	• Th17 cells absent • Decreased IL-17 production
RORγt	Yes	• All IL-17 secreting cells absent
CD4	No	• CD4 cells absent • Increased numbers of CD8 cells
CD8	No	• CD8 cells absent
IL-15Rα	No	• Very few NK or NKT cells • No NK or NKT cell cytolytic ability • Decreased numbers of CD8 cells
$Prkdc^{scid}$	Yes	• Decreased numbers of αβ and γδ T cells • Increased NK cell number and cytolytic ability • Increased numbers of granulocytes

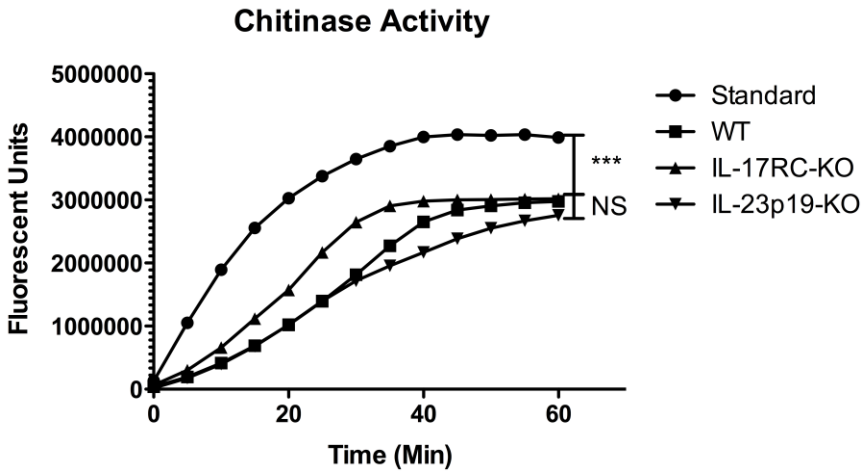
IL-2Rγc	Yes	• Severely immunocompromised
		  ○ γδ T cells, NK cells and NKT cells absent
		  ○ Decreased numbers of CD4 and CD8 cells

$TCR\alpha\beta^{KO}$	No	• Decreased numbers of αβ T cells (CD4 and CD8)
$TCR\gamma\delta^{KO}$	±	• Decreased numbers of γδ T cells

3.2 AMYLASE AND CHITINASE CONCENTRATIONS DO NOT CONTRIBUTE TO CANDIDACIDAL ACTIVITY OF SALIVA

Our laboratory has previously published that the candidacidal activity of saliva from IL-23^{KO} mice was decreased compared to WT both at baseline and on Day 1 of infection (Figure 4A). To further characterize the role of salivary glands in this phenomenon, we performed microarray experiments on mRNA from the submandibular gland to identify any global gene expression changes. The gland was harvested from IL-23^{KO} mice on Day 1 post-infection, and microarray was performed on total mRNA hybridized to Affymetrix Mouse 430(2) chips and analyzed using BRB-ArrayTools (NCI) using an unpaired *t*-test with random variance and a significance threshold of <0.05. A total of 95 genes with $p < 0.05$ and fold-change greater than 2 were found to be altered between IL-23^{KO} and WT mice (data not shown). Surprisingly, no changes were observed in any common immune genes such as chemokines and complement factors, which are observed to be upregulated in Sjögren's syndrome studies (54, 55). Instead, the most strongly downregulated genes in the IL-23^{KO} mice were associated with constitutive mouse salivary gland function, including a highly significant decrease in chitinase and α-amylase activity compared to WT. We therefore hypothesized that differences in expression of these salivary enzymes may provide a partial mechanism for the previous finding by our laboratory that the candidacidal activity of IL-23^{KO} mice and IL-17RA^{KO} mice is decreased compared to WT (20). However, an enzymatic analysis of the amylase and chitinase activity of saliva from IL-23^{KO} mice and IL-17RC^{KO} mice did not show any difference in the activity of these enzymes compared to WT (Figure 9). Therefore, although the expression levels of these genes may differ between IL-23^{KO} mice and WT, there was no measurable functional difference.

A



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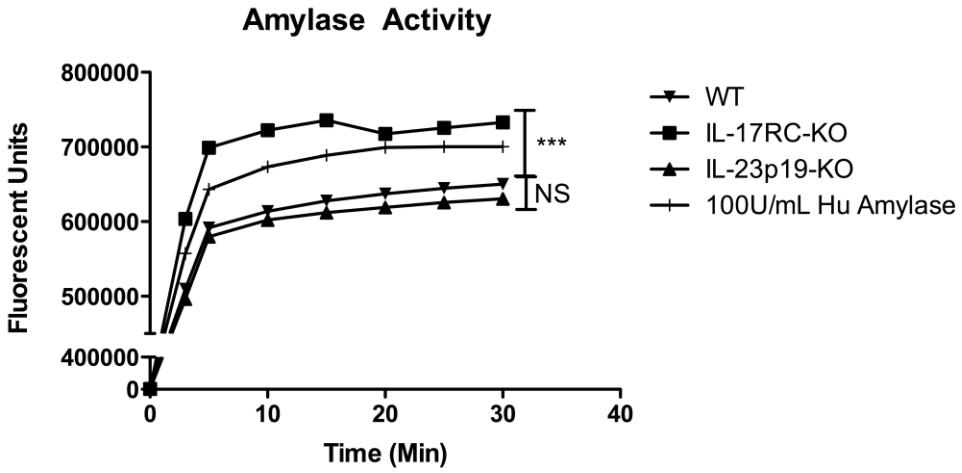

Figure 9: Salivary α-amylase and chitinase levels in IL-23^{KO} and IL-17RA^{KO} mice are similar to WT. **A –** The average concentration of chitinase. **B –** The average concentration of α-amylase. For both experiments, n = 3. ***, < 0.001, significance determined by matched one-way ANOVA. Data representative of two experiments.

3.3 TRANSGENIC MOUSE MODEL OF HIES DEMONSTRATES CLINICAL FEATURES OF HIES, BUT NOT OROPHARYNGEAL CANDIDIASIS

3.3.1 Mouse Model of Hyper-IgE Syndrome Demonstrates Reduced Th17 Differentiation *in vitro*

As described in the introduction to Hyper-IgE syndrome, the majority of patients with AD-HIES carry mutations in the SH2 or DNA-binding domain of one STAT3 allele that prevent the mutant transcription factor from forming functional dimers. To create an animal model that recapitulates this phenomenon, the laboratory of Dr. J.J. O'Shea inserted mutated copies of STAT3 as a transgene into the genome of wild type C57BL/6 embryos (Figure 9A). The inserted STAT3 carried a deletion of Valine at position 463 in the DNA-binding domain, which is a hotspot position for human mutations (36, 56, 57). In addition, the transgene also harbored a partial deletion of the intron downstream of V463. This process yielded two separate lines carrying the mutated transgene, one carrying five copies on the X chromosome (hereafter referred to as Line A), and one carrying two copies on a as yet undetermined somatic chromosome (hereafter referred to as Line B). All data shown in this study were obtained with Line A. Confirmation that progeny generated in our facility carried the transgene was performed by PCR for amplification of the mutated gene as well as the endogenous WT gene (scheme shown in Figure 9B). As expected, all mice carried an endogenous WT gene that was significantly fainter than the transgene in transgene-positive (HIES) mice (Figure 9C).

To further confirm that the mutant transgene decreased STAT3 activity in the HIES mice compared to WT, we examined the ability of HIES mice to generate Th17 cells, which require STAT3 to successfully differentiation. Due to the fact that both alleles of endogenous STAT3 are present in these mice, the mutated transgene STAT3 must compete with wild type STAT3 to induce formation of non-functional mutant:WT heterodimers or mutant:mutant homodimers. Therefore, as expected, HIES mice demonstrated a variable ability to generate Th17 cells (Figure 9D). Although the majority of the HIES mice showed reduced Th17 generation, this was not significant due to the tremendous variability of STAT3 function in these mice.

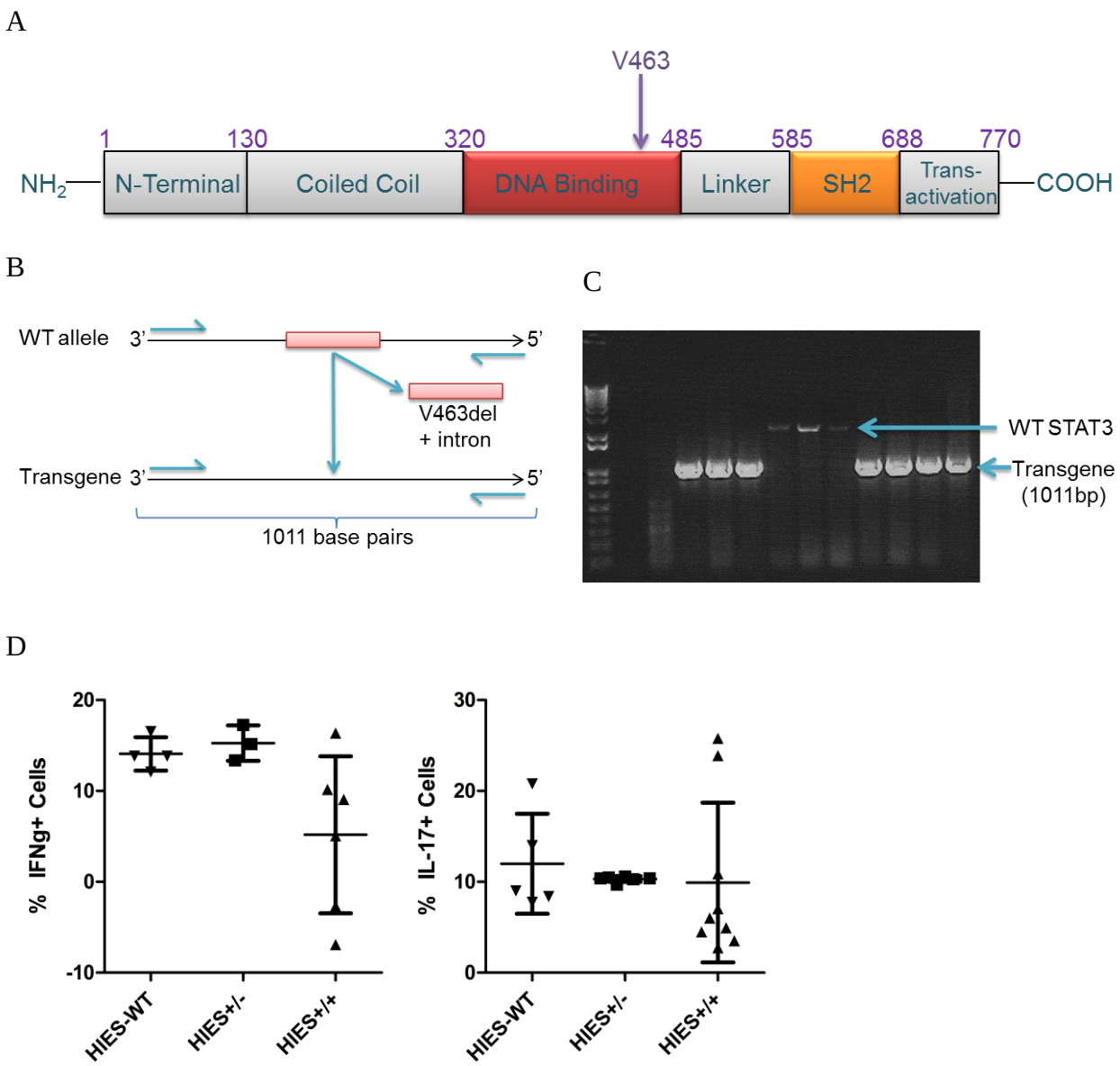

Figure 10: A mouse model of HIES demonstrates a partial defect in Th17 differentiation *in vitro*. **A –** Schematic of the *STAT3* gene indicating the location of the mutation present in the transgene. **B –** Schematic demonstrating the strategy of STAT3 mutation in the transgene. **C –** Representative PCR result of transgenic HIES genotyping (lanes 4-6 and 10-13) versus WT littermate controls (lanes 7-9). **D –** Results of *in vitro* Th17 differentiation of CD4+ splenocytes isolated from spleen. % IFNg+ cells = frequency of IFNγ producing cells induced by Th1 differentiation. % IL-17+ cells = frequency of IL-17 producing cells induced by Th17 differentiation. $HIES^{+/-}$ = X*X; $HIES^{+/+}$ = X*Y

3.3.2 HIES Mice Develop Eczematous Lesions Despite Demonstrating Resistance to Oropharyngeal Candidiasis

Although Hyper-IgE syndrome is associated with a constellation of clinical features, two of the most common clinical manifestations are recurrent OPC and severe eczema. Eczema is often a defining feature that manifests at or around the neonatal period and persists throughout the patient's lifetime (32). Mucosal *Candida albicans* infections also manifest early in life, and may occasionally pre-date development of eczema (33). The recurrent nature of the mucosal candidiasis in these patients commonly requires maintenance antifungal prophylaxis (32).

We examined the susceptibility of both heterozygote ($X^{tg}X$) females and homozygote ($X^{tg}Y$) males to oral challenge with *C. albicans* using our mouse model of OPC. Both $X^{tg}X$ and $X^{tg}Y$ mice exhibited complete resistance, clearing all traces of the fungus, and regaining all lost weight by Day 5 post-infection (Figure 11A and 11B). This may be due to the high variability of Th17 differentiation seen in the splenocytes of these mice, suggesting that the Th17 defect in the mouse model may not be as complete as that seen in HIES patients (Figure 10D). However, the $X^{tg}Y$ mice developed an eczematous rash during the course of the oral challenge (Figure 11C). Histologic analysis of these lesions demonstrated evidence of hyperkeratosis, epidermal hyperplasia and epidermal-dermal separation, indicative of an inflammatory process at the epidermal and dermal layers (Figure 11D). Furthermore, as shown in Figure 11E, the inflammatory skin lesions demonstrated a mixed immune cell infiltrate containing both lymphocytes and granulocytes (eosinophils and neutrophils visible in insets). Staining of these lesions with Periodic Acid Schiff did not demonstrate any *Candida albicans* yeasts or hyphae (data not shown).

In keeping with previously published studies that lack of STAT3 in the skin leads to impaired second hair cycle growth as well as delayed wound repair, uninfected HIES transgenic mice demonstrated both of these phenomenon (58). As shown in Figure 10F, $X^{tg}X$ mice developed hypopigmentation of the hair during the second hair growth cycle. Prior to the second hair cycle, hair appeared identical to that of WT littermate controls (data not shown). In addition, an $X^{tg}Y$ mouse attacked by a littermate WT demonstrated an inability to heal a moderate

thickness skin wound over a period of more than eight weeks, despite topical antibiotic treatment, and isolation from other animals (Figure 11G).

Taken together, these findings suggest that although this HIES mouse model is not appropriate for the study of oropharyngeal candidiasis, these mice do exhibit common skin defects associated with HIES, and may be a good candidate for studying the cutaneous immune response to the Staphylococcal skin infections that plague these patients, as well as for examining the immune response to cutaneous infections by *Candida albicans*.

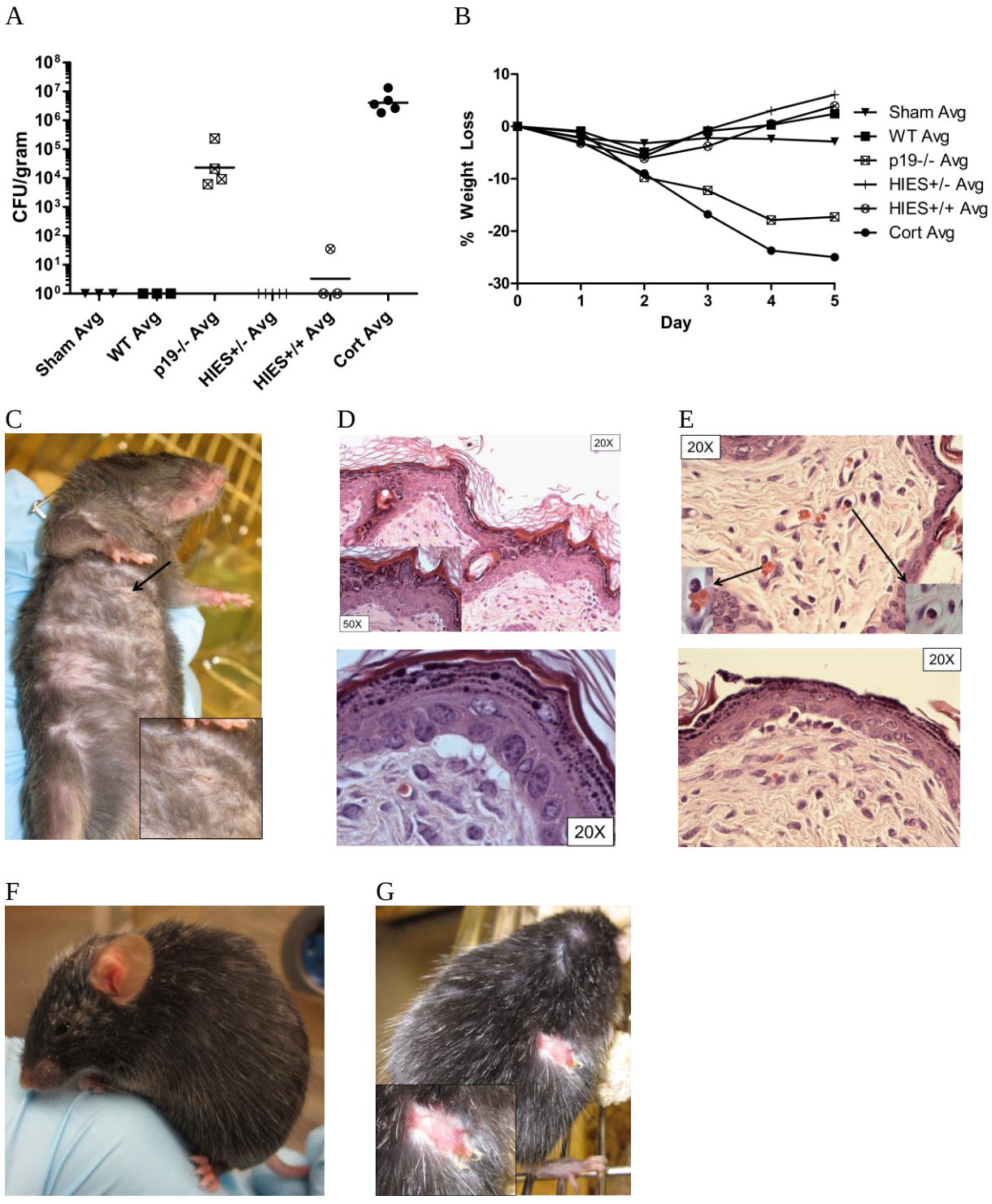

Figure 11: HIES mice are resistant to oropharyngeal candidiasis but demonstrate skin findings similar to both HIES patients and mouse models of STAT3 deficiency. A – Colony forming

units/gram of tongue on Day 5 post-infection with oral *C. albicans.* $HIES^{+/-}$ = X*X; $HIES^{+/+}$ = X*Y**. B –** Percentage weight change compared to Day 0 over the course of the five day infection. **C –** Representative gross lesion present on skin of $X^{tg}Y$ mice infected orally with *C. albicans*. **D and E –** Microscopic images representative of skin changes present in the gross lesion in C. D shows evidence of epidermal hyperplasia and hyperkeratosis (top) as well as epidermal-dermal separation (bottom). E shows evidence of granulocytic (top) and lymphocytic (bottom) immune cell infiltrate. **F –** Gross image of impaired second hair grown in an uninfected $X^{tg}X$ female. **G –** Gross image of a two-month old lesion on an uninfected $X^{tg}Y$ male that shows significantly impaired wound repair.

4.0 CONCLUSIONS

4.1 IL-17 SECRETION IS ESSENTIAL FOR HOST RESPONSE TO *C. ALBICANS* AND IS THEREFORE SECRETED BY MULTIPLE CELL TYPES

Numerous previous studies, including our own, have demonstrated a crucial role for IL-17 in both the oral mucosal and systemic responses to *Candida albicans*. In systemic challenge, both IL-17RA^{KO} and IL-17A^{KO} mice demonstrated reduced survival, and IL-17RA^{KO} mice were also reported to have increased systemic fungal burden and defective neutrophil recruitment to infected organs (19, 59). In the oral cavity, *C. albicans* challenge caused development of overt thrush in IL-23p19, IL-12p40 and IL-17RA mice but not in IL-12p35 or IFNγ knockout mice, demonstrating an essential role for the IL-23/IL-17 axis of immunity in the oral response to *C. albicans* and suggesting that the IL-12/IFNγ pathway is of less importance (20, 22). Furthermore, IL-17 has been shown to be protective in pulmonary challenge by other fungal pathogens, including *Aspergillus fumigatus* and *Pneumocystis jirovecii* (60, 61). In contrast, in a gastric model of candidiasis, IL-17 has been shown to exacerbate disease, suggesting that IL-17 may exhibit location-specific protective effects (62).

In agreement with previous reports, this study demonstrated repeatedly that IL-23^{KO} mice are uniformly susceptible to development of OPC, and in fact can be used as a physiologic positive control in lieu of immunosuppressed cortisone-treated WT mice (Figures 5, 6, 7, 8 and 11). Strikingly, this study further showed that mice deficient in RORγt, who lack all IL-17 producing cells, are more susceptible than IL-23^{KO} mice ($p=0.0082$) to oral challenge with *C. albicans*, further supporting a role for IL-17 (Figure 5C). Although IL-23 is known to enhance IL-17 production as well as expand and potentiate Th17 cells *in vivo*, the cytokine also has other functions. The finding that RORγt^{KO} mice are susceptible supports the hypothesis that loss of

IL-17 secreting cells in IL-23^{KO} mice is responsible for their susceptibility to OPC. Taken together with the previously published data that IL-17RA^{KO} mice are susceptible, these data strongly suggest that IL-17 is essential and irreplaceable for resistance to oral challenge with *C. albicans*. Although we did attempt to demonstrate this phenomenon directly, we were unable to successfully knock down IL-17 *in vivo* using administration of both IL-17 and IL-17 receptor-blocking antibodies (data not shown), perhaps due to difficulty in antibody trafficking into the oral cavity. Nonetheless, IL-17RA^{KO} and IL-17RC^{KO} mice are susceptible to oral candidiasis, consistent with IL-17 dependence for the immune response to OPC (20, 63).

Surprisingly, CD4(stat3^{KO}) mice proved completely resistant to OPC (Figures 5A and 5B), despite the fact that these mice were unable to make any Th17 cells (Figure 5D). Taken together with the fact that RORγt and IL-23^{KO} mice are susceptible to our five day model of OPC, this suggests that the initial immune response to oral challenge to *C. albicans* is mediated by IL-17 production in a cell type(s) other than canonical Th17 cells. Although Th17 cells appear to be dispensable for our model, they may play a major role in memory responses to oral *C. albicans* infections. Data generated from human samples have shown that memory T cells specific for *C. albicans* exhibit a Th17 phenotype as well as mucosal homing molecules (29). Furthermore, patients with IL-17RA mutations or HIES, who are subject to recurrent OPC, show defective Th17 production and abnormal immune responses to *C. albicans* (16, 34).

To determine which cell(s) were responsible for the initial IL-17 production in our model of OPC, we strategically infected a series of knockout mice with defects known to affect IL-17 production in NK, NKT, CD4, CD8 and γδ T cells. We first confirmed our hypothesis that the major initial response to OPC is mediated by an innate cell type and not by T helper cells by examining the susceptibility of CD4 and CD8 knockout mice. Both knockouts were able to completely clear the infection by Day 5 (Figure 6A and 6B), which supported our hypothesis and concurrently confirmed that compensatory Th1 and/or Tc1 (T cytotoxic) responses were not responsible for the clearance of *C. albicans* by CD4(stat3^{KO}) mice.

With the knowledge that neither Th17 nor CD8+ T cells are the major producer of IL-17 in the initial response to OPC, we examined the susceptibility of IL-2Rγc^{KO} mice, which are profoundly immunosuppressed and were therefore expected to demonstrate severe OPC. As expected, these mice were extremely susceptible to disease. Although the oral fungal burden in the IL-2Rγc^{KO} mice was similar to that of both IL-23^{KO} and cortisone-treated WT mice (Figure

6C), these mice had considerably higher morbidity and mortality. IL-2Rγc^{KO} mice were unable to survive past Day 4 due to rapid, excessive weight loss, surpassing even cortisone-treated WT mice in percentage of weight lost over the course of the infection (Figure 6D).

Based on the fact that IL-2Rγc^{KO} mice are deficient in IL-15 and therefore demonstrate reduced numbers of both NK and NKT cells, we next determined the susceptibility to OPC of IL-15Rα^{KO} mice, to either rule in or rule out these cell types. Repeated studies in these mice demonstrated complete resistance to OPC, strongly suggesting that neither NK nor NKT cells play a major role in the initial immune response to OPC (Figure 7A and 7B). However, there remained a distinct possibility that NK and NKT cells do play a role, but that compensatory responses in IL-15Rα^{KO} mice rendered them resistant. Therefore, we used a mouse model of upregulated NK cell number and cytolytic activity, but with severe immunosuppression otherwise (Prkdc^{scid}). SCID mice were susceptible to OPC despite hyperactive NK cell activity, confirming the theory that production of IL-17 by NK and NKT cells is not responsible for buccal mucosa immune responses to *C. albicans* (Figures 7C and 7D).

Given the fact that IL-17 secretion has been demonstrated in γδ T cells, and that these cells are a major factor in mucosal and cutaneous immune responses, we next examined the susceptibility of TCRγδ^{KO} mice in direct comparison with TCRαβ^{KO} mice. Our laboratory had previously published that TCRγδ^{KO} mice are not a major contributor to the immune response to OPC challenge (20), and our data in this study further confirmed that TCRγδ^{KO} mice exhibit variable susceptibility to OPC (Figures 8A and 8B). However, in the context of results generated from all other mouse models tested (Table 1), the variable susceptibility in these mice may not indicate a lack of role for γδ T cells in the initial response to OPC. Rather these data suggest that γδ T cells may act in concert with both the buccal mucosa and other immune cells, and that in the absence of γδ T cells only, compensatory responses exist that render the oral fungal burden in these mice either low or nonexistent.

Taken together, all the susceptibility data generated from specific knockout mice suggests that the immune response to *Candida albicans* at the buccal mucosa surface is IL-17 driven, but that several different cell types are capable of inducing this response (Table 1). Our knockout data suggests that this response is a combination of contributions by lymphocytes, both γδ T cells and αβ T cells (likely Th17, which may facilitate late responses). Therefore in the absence of

either γδ T cells or αβ T cells (as in CD4 or CD8 mice), the immune system mounts a compensatory response to resist *C. albicans* challenge.

Further studies of the molecular mechanisms by which the immune system orchestrates the response to OPC are warranted. Examination of the immune infiltrate present in both the tongue and the buccal mucosa would shed considerable light on which immune cells are found most frequently, and also which are producing IL-17. In addition, using antibodies to eliminate knockout multiple lineages of immune cells may help to determine the interplay between the different IL-17 producing subsets responsible for the response to OPC. It would also be useful to directly eliminate IL-17 from the oral cavity to confirm that the absence of this cytokine confers susceptibility to OPC. And finally, one cannot forget the contribution of the oral mucosa itself to resisting colonization and subsequent infection with *C. albicans*. Our laboratory is currently in the process of generating a K13-Cre mouse that would express Cre solely in the buccal mucosa and tongue, with minimal expression in the esophagus. These mice could then be used to generate buccal specific Cre-Lox-mediated deletions such as crossing the K13-Cre mouse with the stat3$^{fl/fl}$ mouse (48) to specifically induce deletion of STAT3 in the buccal mucosa only.

4.2 SALIVARY ENZYME FUNCTIONALITY DOES NOT DIFFER BETWEEN WT AND IL-23P19 KNOCKOUT MICE AND ARE THEREFORE UNLIKELY TO CONTRIBUTE TO HOST RESISTANCE TO *CANDIDA ALBICANS*

Previous studies from our laboratory have shown that saliva taken from both IL-23^{KO} and IL-17RA^{KO} mice has reduced ability to kill *C. albicans ex vivo* (Figure 4). We concurrently demonstrated that these mice also had decreased gene and protein expression of β-defensin 2, a major antimicrobial peptide that is active against *C. albicans* (20). A preliminary microarray in the submandibular gland of IL-23^{KO} mice further suggested that these mice might have defects in salivary enzyme production compared to WT, specifically in chitinase and α-amylase (data not shown). To determine whether the microarray findings manifested as a difference in functional activity of these enzymes, we developed fluorescent assays to measure chitinase and α-amylase activity directly from saliva harvested from these mice.

In contrast to the microarray data, which demonstrated large differences in the gene expression of chitinase and α-amylase between IL-23^{KO} and WT mice, our functional assays revealed no difference in the activity of these enzymes between IL-23^{KO}, IL-17RC^{KO} and WT mice (data not shown and Figure 9). These data suggest that the concentration of these salivary enzymes does not affect the ability of murine saliva to kill *C. albicans*. Although this was the expected finding for α-amylase, for which the primary function is to begin the degradation process for carbohydrates, chitinase cleaves the polysaccharide chitin, a major component of fungal cell walls including *C. albicans* (64). The fact that the concentration of chitinase does not differ between IL-23^{KO} and WT mice suggests that the major contributors to the candidacidal activity of saliva are likely to be AMPs. This is further supported by the fact that *C. albicans* chitin is shielded on intact fungal cells by mannans, and removal or cleavage of these sugars would be required before chitinase activity would affect the amount of *C. albicans* killing (64). Future studies that examine the specific molecules responsible for the candidacidal activity of saliva would most likely produce meaningful results if they were to examine the concentration and activity of AMPs in the saliva of WT versus knockout mice as well as human HIES subjects. Possible candidates include β-defensins, calprotectins, histatins and other AMPs known to mediate killing of fungal pathogens.

4.3 A STAT3 TRANSGENIC MOUSE CARRYING A DELETION OF V463 DOES NOT FULLY RECAPITULATE HUMAN HYPER IGE SYNDROME, BUT MAY PROVIDE A GOOD MODEL OF HIES-ASSOCIATED SKIN MANIFESTATIONS

Due to variable clinical penetrance, as well as significant variability in STAT3 mutations, HIES patients present with a constellation of clinical manifestations that may or may not be present in any individual patient. However, 100% of patients suffer from eczema and 97% exhibit elevated IgE levels, making these the most common features of HIES (32). Other common manifestations, including mucocutaneous candidiasis (recurrent OPC), recurrent pneumonias and skin infections, and characteristic facial features, are present in greater than 80% of patients, but are by no means definitively present (32).

The transgenic mouse model of HIES described in this study harbored a common human mutation by way of a deletion of the Valine at position 463 along with a partial deletion of the adjacent intron. Although HIES patients with mutations at this site develop both eczema and mucocutaneous candidiasis (36, 57), the mouse model only partially recapitulates the human condition. This may be due to the fact that HIES patients only express one WT allele of STAT3, while the transgenic mice still express two endogenous WT alleles of STAT3. Therefore, despite the presence of multiple copies of the mutant STAT3 transgene on the X chromosome, the endogenous STAT3 concentration of our HIES mice may be higher than that found in HIES patients, and therefore the transgene mutation may not achieve a dominant negative effect whereby STAT3 activity is abrogated by greater than 50%. Increased endogenous STAT3 homodimer formation could explain the variability in Th17 differentiation present in these mice (Figure 10D), and may also explain the difference in clinical manifestations between the mouse model and human HIES patients.

However, despite the fact that these mice are able to resist challenge with *C. albicans* at the oral mucosa, they do exhibit a constellation of skin findings associated with decreased STAT3 activity, including eczematous rash, delayed wound healing and abnormal secondary hair growth (Figure 11). Further study regarding the susceptibility of these mice to both cutaneous *C. albicans* infections as well as Staphylococcal cold abscesses is necessary to determine whether the skin changes described in this study affect the susceptibility of these mice to infection. Even if this mouse model does not show increased susceptibility to skin infections, it may still be a good model for study of eczema in the context of STAT3 deficiency, since HIES mice develop both gross lesions as well as histological changes associated with eczema. Further study is needed to fully characterize the eczematous changes found in the skin of these mice and to identify the immune cell infiltrate in the dermis.

To develop a mouse model of HIES that is susceptible to OPC, similar to HIES patients, the transgenic approach explained in this study could be used to generate mice with other common STAT3 mutations, such as mutations at R382 or V637, which may display a different clinical phenotype than the mice described here. A gene knock-in strategy using a targeted insertion of a mutated gene product into one STAT3 allele is also a potentially robust model that could circumvent the endogenous WT STAT3 expression seen in the HIES transgenic mouse model. Used in conjunction with the specific immune system knockout data generated in this

study (Sections 3.1 and 4.1), a STAT3 model of HIES may help to elicit the specific cell type responsible for IL-17 secretion, or provide insight into new therapeutic targets for OPC treatment and prevention rather than prophylactic antifungals.

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