INTERLEUKIN-17-MEDIATED ADAPTIVE IMMUNITY TO *CANDIDA ALBICANS* IN OROPHARYNGEAL CANDIDIASIS

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Oropharyngeal candidiasis is an opportunistic infection of the oral cavity caused by *Candida albicans,* a fungal member of the human commensal flora that causes disease only under conditions of immunosuppression. In HIV+ individuals, the onset of disease is directly correlated to a decrease in CD4+ T cell counts (<200 cells/mm^3), suggesting that CD4+ T cells are important mediators of anti-*Candida* immunity. Previous studies demonstrated that IL-17 receptor A (IL-17RA) signaling is absolutely required for immunity to OPC. IL-17 is produced by a CD4+ T helper cell subset, Th17, and by a variety of innate immune cell types. Although it has always been assumed that CD4+ T cells are the primary mediators of immunity to *C. albicans,* the relative contribution of Th17 cells in the context of other, innate, sources of IL-17 had never been studied until now. We demonstrate that protective, antigen-specific Th17 cells develop upon secondary infection with *C. albicans* and contribute to fungal clearance from the tongue. Surprisingly, in the absence of CD4+ T cells, compensatory sources of IL-17, such as CD8+ T cells and CD3+CD4-CD8-, protect susceptible hosts from OPC. Our findings have important implications for designing vaccines targeted to immune compromised populations where it is necessary to harness residual immunity.

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LIST OF ABBREVIATIONS

AIDS – Acquired Immune Deficiency Syndrome

AMP – Antimicrobial Peptides

APC – Antigen Presenting Cells

ASC – Apoptosis Associated Speck-Like Protein

BMDC – Bone Marrow Dendritic Cell

CFSE – Carboxyfluorescein diacetate Succinimidyl Ester

CFU – Colony Forming Unit

cLN – cervical Lymph Nodes

CLR – C-type Lectin Receptors

CMC – Chronic Mucocutaneous Candidiasis

DC – Dendritic Cells

DN – Double Negative

FBS – Fetal Bovine Serum

H&E – Hematoxylin & Eosin

HIES – Hyper IgE Syndrome

HK – Heat-Killed

i.p. – intraperitoneally

IACUC – Institutional Animal Care and Use Committee

ICS – Intracellular Cytokine Staining

IL-17RA – Interleukin-17 Receptor A

IL-17RC – Interleukin-17 Receptor C

IL-1R – Interleukin-1 Receptor

iNKT – invariant Natural Killer T Cells

MR – Mannose Receptor

NBF – Normal Buffered Formalin

NLR – Nod-Like Receptors

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

TCR – T Cell Receptor

TDB – Trehalose-6,6-dibehenate

TDM – Trehalose-6,6-dimycolate

Th – T helper

TLR – Toll-Like Receptor

Tregs – Regulatory T Cells

VVC – Vulvovaginal Candidiasis

YNB – Yeast Nitrogen Base

YPD – Yeast Peptone Dextrose

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1.0   INTRODUCTION

1.1 *CANDIDA ALBICANS* AND CANDIDIASIS

C. albicans is a commensal fungus of the human microflora that possesses the ability to undergo morphological transitions from yeast to filamentous forms (pseudohyphae and hyphae) as a result of changes in environmental factors such as temperature. Although *C. albicans* is normally a harmless inhabitant of human mucosal surfaces, it causes disease as a result of breached mucosae, immune suppression or changes in the surrounding microenvironment (Akpan and Morgan, 2002). Candidiasis is classified in two major disease types, disseminated and mucocutaneous. Disseminated disease is a consequence of uncontrolled mucosal infection or damaged mucosal surfaces. It exhibits a mortality rate of approximately 40% (Wisplinghoff et al., 2004) and thus represents a significant health care problem. Chronic mucocutaneous candidiasis (CMC) includes infections of the skin, nails, vaginal tract (vulvovaginal candidiasis, VVC) and oral cavity (oropharyngeal candidiasis, OPC, "thrush"). CMC is mainly a consequence of impaired immune responses due to genetic deficiencies, HIV infection or administration of immunosuppressive drugs (chemotherapy, transplant recipients), although it is known that VVC commonly affects immune competent women.

Of the spectrum of mucocutaneous infections, OPC is particularly interesting due to the strong and well-documented correlation between compromised host immune mechanisms and the onset of disease. In fact, approximately 95% of HIV+ individuals exhibit overt thrush lesions, which consist of pseudomembranous plaques in the tongue, buccal mucosa, palate, periodontal tissues and oropharynx (Akpan and Morgan, 2002; Fidel, 2011). The onset of OPC in HIV+ patients is associated with CD4+ T cell counts below 200 cells/mm^3 (Wright and Johnson, 1997), strongly suggesting that CD4+ T cell

responses are vital for host defense against *C. albicans*. The role of CD4+ T cells in host defense against *C. albicans* has gained considerable attention in recent years and it is the central topic of this dissertation.

1.2   IMMUNITY TO *CANDIDA ALBICANS*

1.2.1   Recognition of fungal pathogen associated molecular patterns (PAMPs) and the priming of adaptive immune responses.

In recent years we have witnessed tremendous advances in our knowledge of how the immune system responds to fungal pathogens. Specifically, several pattern recognition receptor (PRR) pathways of fungal recognition and the type of T cell responses that they prime have been described. Most fungal PRR recognize components of the *C. albicans* cell wall, which is a complex array of layered proteins and carbohydrates (Gow, 2012) (**Figure 1.1**). *Candida albicans* is a dimorphic fungus, existing in yeast (conidia) or hyphal (filamentous) forms. The outer portion of the *Candida* cell wall is largely composed of mannan and manoproteins, and the inner layer is composed of β-(1,3)-glucan and chitin moieties. Expression of cell wall proteins and carbohydrates is significantly altered during the yeast to hyphal transition, which occurs when the fungus invades target organs. The immune system, by virtue of distinct PRRs, can distinguish these fungal forms, in ways that are beginning to be unraveled. Accumulating evidence demonstrates that PRR engagement by *C. albicans* in antigen presenting cells (APCs) results in secretion of specific cytokines including IL-1β, IL-23 and IL-6 (Gow, 2012;

Netea et al., 2008; Romani, 2011). These cytokines in turn promote skewing of activated CD4+ T cells into the Th17 lineage, which express IL-17 (also known as IL-17A), IL-17F and IL-22. IL-17 and IL-17F are closely related cytokines that signal through a common receptor (composed of the IL-17RA and IL-17RC subunits), and IL-17R signaling is clearly crucial for effective anti-*Candida* immunity (Conti and Gaffen, 2010) (**Figure 1.2**).
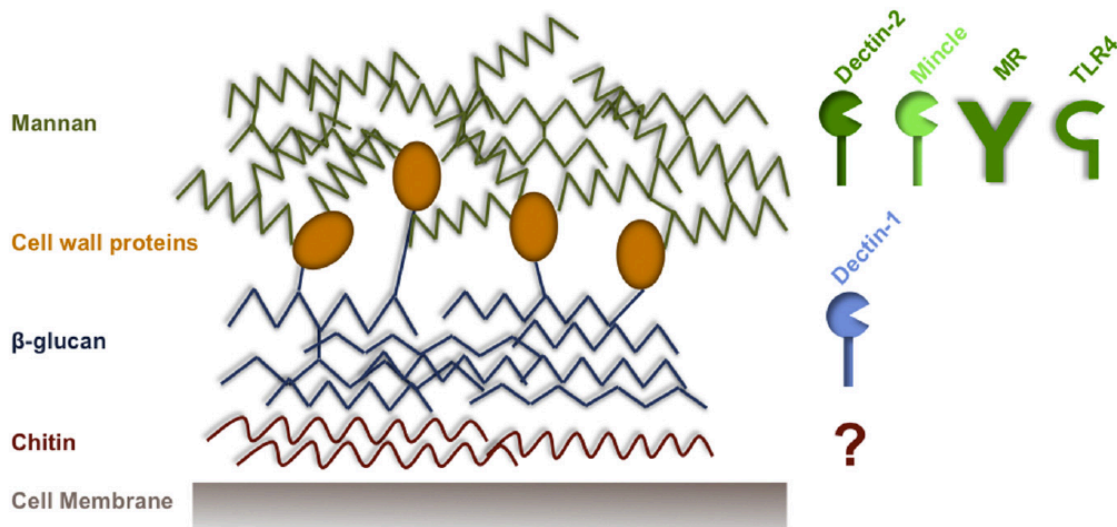
Figure 1.1: *C. albicans* **cell wall and PRRs.** The cell wall of *C. albicans* is composed of proteins and carbohydrates arranged in layers. The outer layer consists of mannoproteins and mannan, which is recognized by a variety of PRR including dectin-2, mincle, MR and TLR4. The middle layer is composed of β-glucan, which is recognized by dectin-1. Finally, the bottom layer is made of chitin for which the PRR has not been identified. Due to its location in the outer layer, mannan is more accessible to the immune system than β-glucan or chitin and it is particularly abundant in the hyphal form. β-glucan is buried underneath the mannan layer but it can be exposed in "bud scars".
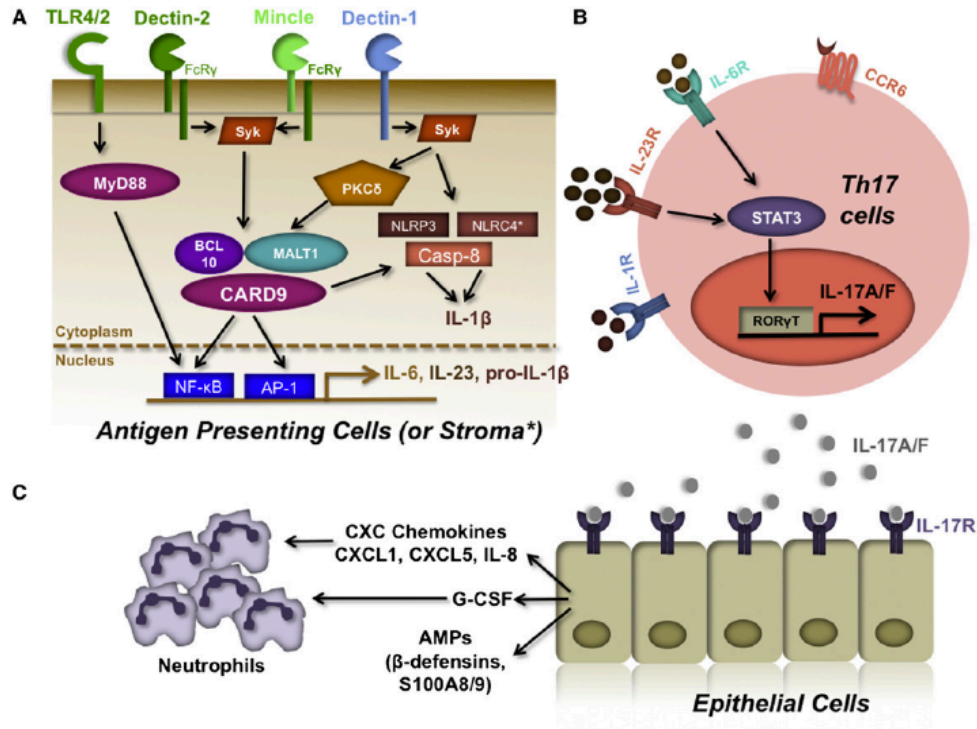
Figure 1.2: *C. albicans* **recognition by antigen presenting cells and the generation of antifungal Th17 responses.** Upon *C. albicans* infection, components of the cell wall are recognized by PRR on the surface of APC. This triggers a series of signaling events including kinases and adaptor molecules like Syk and CARD9, which results in the translocation of NFκB to the nucleus and the production of pro-inflammatory cytokines. Pattern recognition also activates NLRP3 and NLRC4 inflammasomes leading to IL-1β production. Pro-inflammatory cytokines such as IL-6, IL-23 and IL-1β result in the generation of Th17 cells that produce IL-17, which can then bind to its receptor on epithelial cells and trigger the production of antimicrobial peptides and neutrophil recruiting chemokines.

1.2.1.1 Toll-like Receptors (TLR)

Of the Toll-like receptors, TLR2 and TLR4 are the major participants in *C. albicans* recognition. TLR2 binds to phospholipomannans and β-glucan (the major component of yeast zymosan), and acts in combination with dectin-1 to induce pro-inflammatory responses in a variety of *Candida* infection settings (Hise et al., 2009; Netea, 2006; Villamon et al., 2004; Yuan and Wilhelmus, 2010) (**Figure 1.1, 1.2A**). TLR2 has also been shown to suppress inflammatory responses to *Candida* via production of IL-10 and enhanced Treg survival. Accordingly, TLR2$^{-/-}$ mice are more resistant to disseminated candidiasis than WT, supporting a detrimental rather than protective role for this receptor (Netea et al., 2004). On the other hand, TLR4 recognizes *C. albicans* O-linked mannan and stimulates production of the inflammatory cytokine TNFα in human mononuclear cells and murine macrophages. TLR4$^{-/-}$ mice exhibited increased susceptibility to disseminated candidiasis (Netea et al., 2002). Although TLR9, TLR1 and TLR6 are also implicated in recognizing *Candida* pathogen associated molecular patterns (PAMPs), none are required for immunity to this organism (Netea et al., 2008; van de Veerdonk et al., 2008). Mice deficient in MyD88, an adaptor central to TLR signaling, are sensitive to *Candida* infection, which supports a role for either TLRs or alternatively IL-1 family cytokines (Bellocchio et al., 2004). However, humans with MyD88 defects do not appear to be particularly prone to fungal infections (von Bernuth et al., 2008). Thus, other pathways of pattern recognition are important in *C. albicans* immune sensing and the subsequent priming of T helper cell responses. Alternatively, this paradox may indicate a difference in how mice and humans sense this organism.

1.2.1.2 C-type lectin Receptors (CLR)

The CLR have garnered considerable attention in the context of *Candida*, and appear to be comparatively more critical than TLR for immunity in both mouse models and humans (Saijo and Iwakura, 2011; Vautier et al., 2010). The best-characterized CLR with respect to *Candida* are dectin-1, dectin-2 and mincle **(Figure 1.2)**. Although details of their respective signaling pathways are still being elucidated, they all appear to mediate signaling through the kinase Syk1, the adaptors CARD9/Bcl-10/MALT1 and the NF-κB and Ras/Raf-1 pathways (Gow, 2012; Willment and Brown, 2008). Dectin-2 and Mincle form heteromeric complexes with the FcγR, whereas Dectin-1 does not engage this subunit. Recently, the kinase PKCδ was shown to be activated by dectin-1 and induce phosphorylation of CARD9. Both CARD9-/- and PKCδ-/- mice are susceptible to disseminated candidiasis (LeibundGut-Landmann et al., 2007; Strasser et al., 2012). The CLR DC-SIGN in human dendritic cells (DCs) is involved in mediating *Candida* uptake via recognition of N-linked mannan (Cambi et al., 2008; Takahara et al., 2012). Although the role of DC-SIGN in the generation of Th17 responses during *C. albicans* infection is unclear, it appears to inhibit dectin-1-dependent Th17 generation and instead favor Th1 responses in a model of tuberculosis (Zenaro et al., 2009). Similarly, the lectin Galectin 3 also recognizes *Candida* (Brown, 2010; Gow, 2012; Netea et al., 2008). Although the specific contribution of this receptor to antifungal immunity is not well defined, a recent study showed that Galectin 3 associates with dectin-1 and contributes to TNFα production in macrophages (Esteban et al., 2011). Exactly how signals from these disparate CLRs are integrated is still not fully understood.

Dectin-1 recognizes the β-(1,3)-glucan, which is usually buried underneath a layer of cell wall proteins and mannan moieties, posing an issue of accessibility for innate immune cells **(Figure 1.1)**. Nevertheless, β-glucan is exposed in bud scars that are revealed during the process of hyphal transition, which facilitates *Candida* recognition and may be the essential signal that alerts the host of a transition from fungal colonization to infection (Netea et al., 2008). Consistently, dectin-1-deficient mice exhibit impaired production of inflammatory cytokines such as IL-6 and granulocyte-colony stimulating factor (G-CSF) (Taylor et al., 2007), which drive Th17 differentiation and are induced upon IL-17 signaling (Gaffen, 2009), respectively **(Figure 1.2)**. Furthermore, β-glucan stimulation of bone marrow dendritic cells (BMDCs) promotes skewing of Th17 cell differentiation, thus linking CLR signaling and activation of a Th17 response (LeibundGut-Landmann et al., 2007).

However, the role of dectin-1 in fungal host defense remains a topic of debate. Whereas one study showed that dectin-1-/- mice showed increased susceptibility to disseminated candidiasis (Saijo et al., 2007; Taylor et al., 2007) another report found that dectin-1-/- mice were resistant. Intriguingly, in the study by Saijo *et al.,* dectin-1-/- mice were susceptible to *Pneumocystis carinii*, suggesting that the role of dectin-1 in antifungal immunity may be pathogen-specific. In addition to promoting Th17 responses, dectin-1 signaling appears to play a role in balancing the frequencies of Th1 and Th17 cells. Specifically, the absence of dectin-1 during lung infection with *Aspergillus fumigatus* causes reduced production of IFNγ and T-bet, a transcription factor that controls Th1 differentiation, resulting in decreased Th1 responses and correspondingly enhanced Th17 differentiation (Rivera, 2011). Therefore, β-glucan

recognition by dectin-1 shapes the overall nature of antifungal CD4+ T helper responses. As described in detail below, dectin-1-deficient humans with chronic mucocutaneous candidiasis (CMC) have been identified, supporting the idea that dectin-1 is a bona fide recognition element for *Candida* **(Table 1.1).** However, disease in these patients was generally mild, and the mutation was later found to be a fairly common polymorphism linked to *Candida* colonization in transplant recipients (Plantinga et al., 2009). Thus, PRRs other than dectin-1 probably play central roles in immunity to *Candida*.

Dectin-2 recognizes N-linked mannan sugars, which are localized in the exterior layer of the yeast cell wall (Robinson et al., 2009), and appears to be especially important in recognition of hyphae (Bi et al., 2010). Similar to dectin-1, stimulation of BMDCs via dectin-2 triggers Th17 differentiation. *C. albicans*-specific Th17 cells are diminished more dramatically in the absence of dectin-2 than in the absence of dectin-1 (Robinson et al., 2009). However, not all studies of dectin-2 in antifungal host defense are consistent. One report showed that antibody blockade of dectin-2 in dectin-1-/- mice led to reduced IL-17 production during systemic *Candida* infections. Surprisingly, however, diminished IL-17 production did not correlate with disseminated disease as assessed by kidney fungal burden (Robinson et al., 2009). In contrast, dectin 2-/- mice were found to be more susceptible to disseminated candidiasis than WT mice (Saijo et al., 2010). The basis for these discrepancies was attributed to different observation periods and disease kinetics, but may also indicate that additional elements are involved in host immunity to *Candida*. While dectin-1 and dectin-2 recognize different components of the *Candida* cell wall and thus may preferentially "see" yeast versus

hyphal forms of the microbe, *in vivo* studies have not yet fully clarified which CLR is more important. Ultimately, it is likely that signaling through a multiplicity of PRRs is needed to develop an optimal immune response.

In addition to dectins, mincle and the mannose receptor (MR) recognize mannan carbohydrates from *Candida* **(Figure 1.1).** In human peripheral blood mononuclear cells (PBMC), mannan was found to induce more IL-17 than other fungal PAMP such as β-glucan and chitin (van de Veerdonk et al., 2009), perhaps indicating a dominant role for MR in this process. Interestingly, this study also demonstrated MR-dependent production of IL-17 in cells from dectin-1-deficient patients, supporting the idea that stimulation of MR by *C. albicans* is potentially a primary pathway for the generation of antigen-specific Th17 responses. Mincle binds to the mycobacterial component Trehalose-6,6-dimycolate (TDM) and its synthetic analogue Trehalose-6,6-dibehenate (TDB), and induces Th1 and Th17 responses in a Syk- and CARD9-dependent manner (Ishikawa et al., 2009; Schoenen et al., 2010; Werninghaus et al., 2009). This suggests that fungal cell wall components such as α-mannans may induce Th17 responses via mincle. Interestingly, whereas mincle-deficient mice are susceptible to disseminated candidiasis (Wells et al., 2008), MR-deficient mice are resistant (Lee, 2003), arguing that the MR is redundant with CLRs in terms of mediating anti-*Candida* immunity.

Table 1.1: Human genetic deficiencies associated with CMC.

Gene product	Disease	Impact on Th17/IL-17	References
Dectin-1	CMC	Reduced Th17 frequency	(Ferwerda et al., 2009)
CARD9	CMC; disseminated candidiasis	Impaired dectin-1 signaling. Reduced Th17 frequency	(Glocker et al., 2009)
STAT 3	Autosomal dominant Hyper I.e. (Job's) Syndrome; CMC	Impaired IL-6, IL-21, IL-22 and Il-23 signaling. Reduced Th17 frequency	(Ma et al., 2008; Milner et al., 2008)
TYK 2	Autosomal recessive Hyper IgE (Job's) Syndrome; CMC	Defective IL-23 signaling. Reduced Th17 frequency.	(Minegishi, 2006)
IL-17RA	CMC	Complete IL-17RA deficiency. Abolished response to IL-17A/F	(Puel, 2011)
IL-17F	CMC	Impaired signaling through IL-17A/F	(Puel, 2011)
STAT-1	CMC	Increased response to IFNα/β and IL-27. Reduced Th17 frequency.	(Liu et al., 2011; Veerdonk, 2011)
AIRE	Autoimmune Polyendocrinopathy Syndrome-1; CMC	Neutralizing antibodies against IL-17A/F and IL-22	(Kisand et al., 2010; Puel et al., 2010)

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1.2.1.3 The inflammasome

The intracellular inflammasome has emerged as another PRR in antifungal host defense. Composed of cytosolic Nod-like receptors (NLRs), notably NLRP1, NLRP3 and NLRC4, complexed with several adaptors and proteases, the inflammasome activates caspase-1 and thereby permits cleavage and secretion of the proinflammatory cytokines IL-1β and IL-18. While neither the cytosolic receptors NOD1 nor NOD2 are

required for *Candida* recognition (van der Graaf et al., 2006), inflammasomes are involved. Mice deficient in the IL-1 receptor (IL-1R) or apoptosis associated speck-like protein (ASC), an essential subunit of the inflammasome, exhibit increased oral fungal burdens in a model of oral mucosal candidiasis (Hise et al., 2009). Fungal dissemination in this model was even more pronounced than mucosal infection. Consistent with this concept, NLRP3-/- mice are susceptible to disseminated candidiasis as well as mucosal disease (Gross et al., 2009). Elegant bone marrow chimera experiments confirmed the role of the NLRP3 inflammasome in preventing *Candida* dissemination, and further demonstrated that the NLRC4 inflammasome compartment was also involved in oral *C. albicans* host infections (Tomalka et al., 2011). Notably, NLRP3 is required in hematopoietic cells, whereas NLRC4 functions at the level of the mucosal stroma. Moreover, both NLRP3 and NLRC4 deficiencies resulted in decreased expression of IL-17, IL-17F and also one of the IL-17 receptor subunits (IL-17RA), directly linking the inflammasomes and IL-17 (Tomalka et al., 2011). Furthermore, BMDCs from ASC-/- and caspase-1-/- mice exhibited impaired production of IL-1β and IL-18 following *Candida* exposure, which are important for Th17 and Th1 development, respectively. Indeed, splenocytes from IL-1β- and IL-18-deficient mice exhibited impaired production of IL-17 and IFNγ in response to stimulation from *Candida* (van de Veerdonk et al., 2011). The development of protective Th17 responses via inflammasome activation resulted from the recognition of *C. albicans* hyphae by human macrophages. The yeast form did not activate the inflammasome, demonstrating that this pathway is likely important for discriminating between colonizing yeast and invasive hyphae (Cheng et al., 2011).

Recently, another member of the NLR family, NLRP10, was demonstrated to play a role in host defense against disseminated candidiasis (Joly et al., 2012). NLRP10-mediated anti-*Candida* immunity depended on induction of protective T cell responses, presumably Th1- and Th17- mediated as indicated by decreased IFNγ and IL-17 production from NLRP10-/- splenocytes re-stimulated *in vitro* with HK *C. albicans.* The absence of NLRP10 did not affect the production of IL-1β, indicating that *C. albicans* recognition by NLRP10 does not affect IL-1β production by NLRP3 and NLRC4 inflammasomes.

In addition to the role of the NLRP3 and NLRC4 inflammasomes in processing pro-IL-1β*,* a recent study identified dectin-1-dependent activation of a non-canonical caspase-8 inflammasome (Gringhuis et al., 2012). Dectin-1 engagement resulted in the activation of Syk and the transcription of the IL-1β gene through the CARD9-Bcl10-MALT1 complex **(Figure 1.2)**. Recruitment of MALT1-caspase-8 and ASC to this scaffold resulted in processing of pro-IL-1β to mature IL-1β. The activation of this non-canonical pathway did not require *C. albicans* internalization, whereas activation of NLRP3 inflammasome was completely dependent on internalization. Interestingly, some *C. albicans* strains trigger IL-1β production primarily via caspase-8 while others also activate the NLRP3 inflammasome, suggesting that the ligand for NLRP3 is not present in all fungi. It would be of interest to determine the role of the caspase-8 inflammasome in the development of Th17 responses, but that was not addressed in this study.

Taken together, the priming of host-protective Th17 responses is likely a combined effort of multiple PRR pathways that recognize different components of *C.*

albicans, and trigger the production of cytokines that predominantly direct Th17 differentiation. Nuances of how different fungal PAMP trigger different PRR is an area that requires more development, and subtle alterations of the host response may ensue depending on the specific substrain of *Candida* that is involved and the anatomic site of infection.

1.2.2 T cell responses to *C. albicans*.

1.2.2.1   Life before IL-17 was all about Th1 cells

HIV+ individuals are exquisitely susceptible to many opportunistic fungal pathogens, including oral thrush*.* Importantly, the development of opportunistic infections in HIV patients strongly correlates with loss of CD4+ T lymphocytes, indicating that cytokines produced by CD4+ T cells are important in immunity to *Candida* (Akpan and Morgan, 2002). Early studies aimed at elucidating the role of CD4+ T cells in host defense against *C. albicans* employed a mouse model of gastric infection. In this setting, both IFNγ- and IL-5-producing CD4+ T cells, indicative of Th1 and Th2, respectively, were found in Peyer's Patches and mesenteric lymph nodes, and corresponded with fungal clearance. Furthermore, neutralization of IL-4 resulted in increased IFNγ production and enhanced yeast clearance (Cenci et al., 1995). These data were interpreted to mean that protective antifungal immunity is attributable to Th1 cells, even though hallmark cytokines of both Th1 and Th2 lineages were produced during infection (Cenci et al., 1998; Cenci et al., 1995). Since these studies occurred prior to the recognition of the

Th17 population, the alternative interpretation that non-Th1/Th2 lineages might be involved was not considered.

Studies in a mouse model of OPC showed that nude (T cell deficient) mice were susceptible to infection, but could be protected by transfer of CD4+ T cells (Farah et al., 2002a). Th1-related cytokines, including IFNγ and TNFα, but not Th2 cytokines were expressed in the oral tissue, consistent with a Th1-biased response (Farah et al., 2002b). Interestingly, however, depletion of CD4 or CD8 T cells in a susceptible mouse strain did not exacerbate oral colonization with *Candida* (Ashman et al., 2003), although adoptive transfer of *Candida*-specific T cells did induce protection from a disseminated challenge (Sieck et al., 1993). Thus, both innate cells and CD4 cells were implicated in immunity to OPC, although the specific subset and cytokines were still not well clarified.

1.2.2.2 Life after IL-17 is all about Th17 cells

The discovery of Th17 cells (Harrington et al., 2005) and the role of IL-23 in their homeostasis (McGeachy et al., 2009) have contributed tremendously to our understanding of the host immune responses to fungal pathogens. Prior to this important discovery, immunity to most pathogens was attributed to either Th1 or Th2 cells. Since Th2 responses were thought to promote fungal pathogenesis, investigative efforts focused on elucidating how Th1 cells defended the host against infection. Most of these studies were conducted using mice lacking p40, which is a subunit of IL-12, an important cytokine for the differentiation of Th1 cells. However, it was later demonstrated that IL-12 shares the p40 subunit with IL-23 (Ghilardi and Ouyang, 2007),

which is paramount for the expansion and pathogenicity of Th17 cells (Lee et al., 2012; McGeachy et al., 2009). Therefore, antifungal immune responses initially attributed to Th1 cells may have in fact been Th17 driven.

A role for the IL-17 axis in antifungal host defense was first shown in 2004, in which IL-17RA-deficient mice inoculated i.v. with *C. albicans* exhibited decreased survival and increased kidney fungal burden compared to WT counterparts (Huang et al., 2004). Although disputed in one report (Zelante et al., 2007), most data are consistent with a protective role for IL-17 in systemic candidemia. Furthermore, an experimental vaccine containing the *C. albicans* adhesin protein Als3p and aluminium hydroxide as an adjuvant conferred protection against systemic candidiasis via induction of both Th17 and Th1 responses (Lin, 2009).

In a model of OPC, mice lacking Th1 effector cytokines IFNγ or TNFα were resistant to oral infection, yet mice lacking IL-12p40 were found to be susceptible (Farah et al., 2006), suggesting a role for protective IL-23-dependent pathways. In a direct test of this hypothesis, our laboratory showed that IL-23p19-/- mice subjected to OPC exhibited overt thrush lesions and elevated fungal burdens whereas IL-12p35-/- mice did not. High susceptibility was also observed in IL-17RA-/- and IL-17RC-/- mice, implicating the IL-17 pathway directly (Conti et al., 2009; Ho et al., 2010). Furthermore, in extended time courses, IL-17RA-/- mice never recovered from *Candida* infection, whereas IL-12p35-/- mice fully cleared the microbe, albeit delayed compared to WT mice.

Parallel findings were made in mouse models of dermal candidiasis, in which IL-17 and IL-23 but not IL-12 were essential for immunity to *Candida* (Kagami et al., 2010).

A detailed study of skin-resident DC subsets showed that Th17 cells against *Candida* are generated specifically by presentation from Langerhans cells. Interestingly, generation of Th17 cells is blocked by signals from Langerin+ dermal DCs in favor of Th1 cells (Igyarto et al., 2011). In a vaginal candidiasis model, treating with halofuginone, which specifically blocks Th17 differentiation, resulted in a profound decrease in IL-17 production that correlated with an increase in fungal burden (Pietrella et al., 2011). Thus, similar to disseminated candidemia, mouse models of skin and mucosal candidiasis implicate IL-17 and IL-23 in immunity to *Candida*.

In addition to Th17 cells, there is an unexpected interplay between Th17 cells and regulatory T cells (Tregs), a subset of immunoregulatory T cells, suggesting both are required for effective host responses to *Candida*. Th17 and Treg cells both arise from signals from TGFβ (although this is still somewhat controversial) (Ghoreschi et al., 2010), with IL-6 and IL-1β providing the inflammatory switch that favors Th17 cells, and IL-2 driving Tregs and preventing Th17 differentiation (Laurence et al., 2007; Yang et al., 2011). Rag-/- mice, which lack both T and B cells, are extremely susceptible to OPC. However, transfer of naïve or Th17-polarized T effector cells alone is insufficient to mediate host defense to OPC; rather, co-transfer of regulatory T cells resulted in enhanced Th17 responses that were needed to prevent development of OPC. Mechanistically, this was shown to be due to a sequestration of IL-2 by Tregs via the high affinity IL-2 receptor complex. Consistently, depletion of Tregs in mice increases susceptibility to OPC (Pandiyan et al., 2011). This finding is in agreement with an earlier report that demonstrated a positive correlation between the levels of IL-17 produced by curdlan-stimulated BMDCs and the ratio of Treg to T effector cells

(LeibundGut-Landmann et al., 2007). Interestingly, stimulation of BMDC with curdlan triggered conversion of Foxp3+IL-17- cells into IL-17-expressing Foxp3+ cells (Osorio et al., 2008), suggesting that dectin-1 enhances a Th17 response by converting Tregs. Thus Tregs play a previously unappreciated protective role in inflammatory responses during *C. albicans* infection.

Intriguingly, *C. albicans* appears to actively target the IL-17 pathway, presumably as an evasive strategy. Whereas heat-killed *Candida* stimulated PBMC secrete IL-17, co-culture with live *Candida* strongly suppresses IL-17 production. This factor is apparently soluble (and thus far, unidentified), because live *Candida* exerted this suppressive effect even when separated from PMBC in a trans-well system. The mechanism appears to be via regulation of tryptophan metabolism. Specifically, suppression of IL-17 observed in co-culture was associated with reduced L-kynurenine (an indoleamine 2,3-dioxygenase [IDO]-dependent metabolite, representing one pathway of tryptophan metabolism) and increased 5-hydroxytryptophan (representing the alternate pathway). Consistently, ectopic application of 5-hydroxytryptophan inhibited IL-17 production (Cheng et al., 2010). Another recent report suggests that IL-17 might directly bind to *Candida* and induce nutrient starvation conditions in the organism (Zelante et al., 2012). Thus, there is still much to learn about the intricate interactions occurring between *Candida* and its host.

In addition to IL-17A, other Th17-derived cytokines such as IL-17F and IL-22 may participate in anti-*Candida* immunity. In contrast to IL-17A-/- mice, IL-17F knockouts are resistant to systemic candidiasis (Saijo et al., 2010), although the role of IL-17F in experimental mucosal candidiasis has not yet been evaluated. As noted in **Table 1.1**,

dominant negative mutations in IL-17F in humans are linked to CMC, although the mutations affect IL-17A as well as IL-17F signaling (Puel, 2011). Surprisingly, IL-22-/- mice inoculated orally with *C. albicans* had a significantly lower fungal burden than IL-17RA-/- or IL-23-/- mice, despite the observation that IL-22 mRNA is strongly induced in WT mice following oral *Candida* infection (Conti et al., 2009). Similarly, in dermal candidiasis there was no major role for IL-22 (Kagami et al., 2010). However, De Luca *et al.* reported that IL-22-/- mice are susceptible to both systemic and gastric candidiasis, due in part to impaired production of antimicrobial peptides (AMPs) such as S100A8, S100A9, RegIIIβ and RegIIIγ. In addition, blockade of IFNγ in IL-22$^{-/-}$ mice resulted in fungal dissemination, suggesting that IL-22 together with IFNγ may provide a first line of defense in preventing dissemination from the gastric mucosa (De Luca et al., 2010). In a recent study where human keratinocytes were infected *in vitro* with *C. albicans*, IL-22 in combination with TNFα led to decreased cell death and epidermal damage caused by infection (Eyerich et al., 2011). However, IL-22 is impaired in several human CMC syndromes **(Table 1.1)**, and thus its role in human candidiasis cannot be ruled out.

Most of the studies in experimental candidiasis have described a protective role for IL-17 in host responses to *C. albicans.* However, a detrimental role of this cytokine has also been reported, specifically in a model of gastric candidiasis. IL-23p19-/- and IL-12/IL-23p40-/- mice inoculated intragastrically with *C. albicans* showed 100% survival over a 2 week period, whereas only 25% of IL-12p35-/- mice survived. Furthermore, expression of IL-12p70 and IFNγ in stomach correlated with protection in IL-23-/- mice, whereas levels of IL-23p19 and IL-17 were linked to severity of disease (Zelante et al.,

2007). *Candida* does colonize the gut, but the clinical relevance of the gastric candidiasis disease model is unclear. Although some cases of gastric candidiasis have been reported, they are extremely rare due to the low pH and inhospitable conditions of the stomach (Filler, 2012). Regardless, these studies indicate that lessons of immunity in the gut mucosa cannot necessarily be applied to other sites.

1.3 "EXPERIMENTS OF NATURE": HUMAN GENETIC DEFICIENCIES ASSOCIATED WITH CHRONIC MUCOCUTANEOUS CANDIDIASIS.

The identification of Th17 cells and CLR as elements of anti-fungal immunity facilitated characterization of human genetic deficiencies underlying development of CMC, either in isolation (CMC disease) or in the context of an immune disorder **(Table 1.1).** The most direct link of IL-17 to the etiology of CMC disease comes from a recent report describing rare human pedigrees with mutations in the IL-17 signaling axis. One individual had an autosomal recessive mutation in IL-17RA, and cells from this patient did not express the receptor and hence were refractory to IL-17 signaling. Another cohort exhibited an autosomal dominant mutation in IL-17F that prevents signaling through IL-17A, IL-17F and the IL-17A/F heterodimer (Puel, 2011). In separate studies, gain of function mutations in signal transducer and activator of transcription (STAT) 1 were identified as causes of CMC disease, and were associated with reduced Th17 frequency (Liu et al., 2011; Veerdonk, 2011). It is not fully clear why this STAT1 mutation causes CMC and reduced Th17 cell numbers, but STAT1-activating cytokines such as IFNα/β, IFNγ and IL-27 are all inhibitors of Th17 differentiation, and accordingly

Th17 cells appear to be abnormally restrained in these patients.

In scenarios where CMC is present in conjunction with other infections and/or inflammatory defects, identifying a single cause for recurrent candidiasis poses a challenge. Strikingly, in many cases defects in some component of Th17 differentiation or the pattern recognition pathways that promote it have been linked to disease. For example, Hyper IgE (Job's) Syndrome (HIES) patients suffer from recurrent *Staphylococcus aureus* and *C. albicans* infections, and exhibit a Th17 deficiency due to dominant negative mutations in STAT3 (Ma et al., 2008; Milner et al., 2008; Minegishi et al., 2007)(Ma et al., 2008; Milner et al., 2008; Minegishi et al., 2007) STAT3 is critical for Th17 differentiation in mice (Chen et al., 2006; Yang et al., 2007), as it lies is downstream of signaling by IL-23, IL-21 and IL-6 (all involved in induction and maintenance of Th17 cells) as well as IL-22 (produced by Th17 cells). Although a failure to differentiate Th17 cells is one obvious cause of their susceptibility to *Candida*, these patients also have impaired antifungal activity of saliva (Conti et al., 2011), and their keratinocytes show defective responses to Th17 cytokines (Minegishi et al., 2007).

Mutations in the PRR pathway have also been identified that cause CMC, reinforcing results from knockout mouse studies. Some CMC patients show deficiencies in CARD9 or dectin-1. Notably, the dectin-1 mutation appears to be a polymorphism found in a population-wide search in individuals from both Europe and Africa and is associated with increased *Candida* colonization in immunosuppressed hematopoietic stem cell transplant recipients (Ferwerda et al., 2009; Glocker et al., 2009; Plantinga et al., 2009).

Another cohort of patients with CMC have autoimmune polyendocrinopathy

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syndrome -1, characterized by genetic defects in central tolerance due to mutations the transcription factor *AIRE* (Brown, 2010). The cause of CMC in APS-1 was enigmatic, until it was discovered in 2010 that these patients produce neutralizing autoantibodies against Th17 cytokines, namely IL-17A, IL-17F and IL-22 (Kisand et al., 2010; Puel et al.). These observations, together with the fact that the majority of *C. albicans*-specific T cells in human peripheral blood exhibit a classical Th17 phenotype (Acosta-Rodriguez et al., 2007), strongly support a crucial role for Th17 cells in immunity to *C. albicans.*

2.0   CHAPTER TWO: METHODS

2.1   MICE

All mice used in these studies were ordered from The Jackson Laboratories (Bar Harbor, ME) in the C57BL/6 background unless otherwise indicated. IL-23 (p19)-/- mice were bred in-house and acquired from Genentech (San Francisco, CA). In all experiments, mice were matched according to gender and age, which ranged from 5 to 6 weeks. Animal protocols were approved by the University of Pittsburgh IACUC, and adhered to guidelines in the Guide for the Care and Use of Laboratory Animals of the NIH.

2.2   *C. ALBICANS* GROWTH AND CULTURE CONDITIONS

For infection **(Figure 2.1)** and *in-vitro* cultures **(Figure 2.2)** *C. albicans* (strains CAF2-1, CAYC2QTPU1 and CJN1348) was grown overnight (approximately 16 hours) in YPD broth at 30°C with continuous agitation. For quantification of fungal burden in the tongue following infection, tissue homogenates were plated in YPD agar containing ampicillin and incubated at 30°C for 48 hours.

2.3   RECALL MODEL OF OPC

Mice were inoculated sublingually with *C. albicans* by placing a 0.0025g cotton ball saturated in a 2×10^7 CFU/ml cell suspension underneath the tongue for 75 minutes. Sham infections were performed in parallel using a cotton ball saturated in PBS. This procedure was performed under anesthesia, which consisted of 100 mg/kg Ketamine and 10 mg/kg Xylazine mixed in saline (0.9% NaCl). Mice received 1ml of saline both before and after the 75 minutes inoculation period. Secondary infection was performed 6 weeks later exactly as described above. At days 1,2,3 and 5 post re-challenge, one half of the tongue was homogenize by mechanical disruption using a GentleMACS Dissociator (Miltenyi Biotec) and plated in triplicate for colony enumeration. The other half of the tongue was preserved in RNA later (Ambion) and frozen at -80°C for subsequent RNA extraction and real-time PCR. The cLN were also harvested and homogenized manually by mechanical disruption. The resulting single cell suspension was analyzed by flow cytometry or cultured *in vitro* with heat-killed (HK) *C. albicans* for quantification of cytokine levels by ELISA (eBioscience). A schematic of this infection model is presented in **Figure 2.1.**
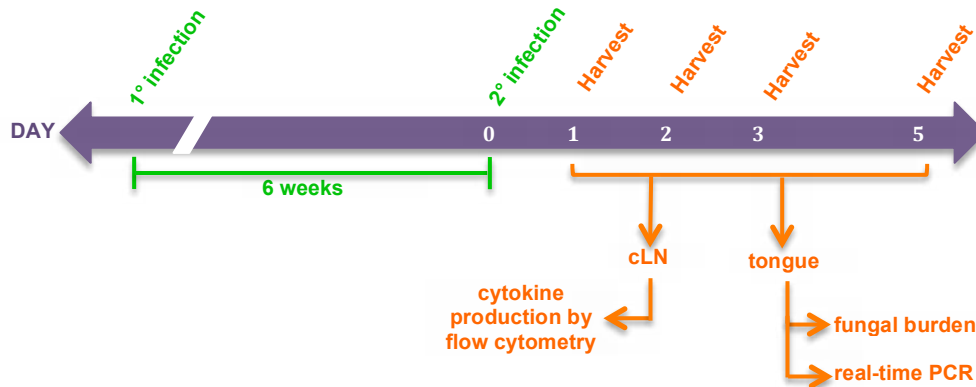
Figure 2.1: Recall model of OPC. Mice were infected two consecutive times with the same dose of *C. albicas* (2 x 107 CFU/ml) leaving an interval of 6 weeks in between infections. At days 1-5 after re-challenge the indicated tissues were harvested for assessment of fungal burden and cytokine production.

2.4   IN VITRO CULTURE OF CERVICAL LYMPH NODE CELLS WITH *C. ALBICANS* AND QUANTIFICATION OF CYTOKINES

One million cLN cells were plated in flat-bottom 24-well plates and stimulated with 2 x 10^6 HK *C. albicans* yeasts or 5-10µg/ml pALS (NH_2- KGLNDWNYPVSSESFSYT - COOH) in the presence or absence of 5µg/ml anti-CD4 (clone RM4-5, BD Biosciences) or 10µg/ml anti-MHC class II blocking antibodies **(Figure 2.2)**. Co-cultures were incubated at 37°C and 5% CO_2 for 5 days. Following incubation, culture supernatants were harvested and analyzed for IL-17 and IFNγ levels by ELISA according to the

manufacturer's instructions (eBioscience). HK *C. albicans* was prepared by boiling 1ml of overnight culture for 45 minutes. These experiments were performed in serum-free AIM V media (GIBCO Invitrogen) containing 1X β-Mercaptoethanol (1000X concentrated, 55mM, GIBCO Invitrogen) and 20U/ml of IL-2.
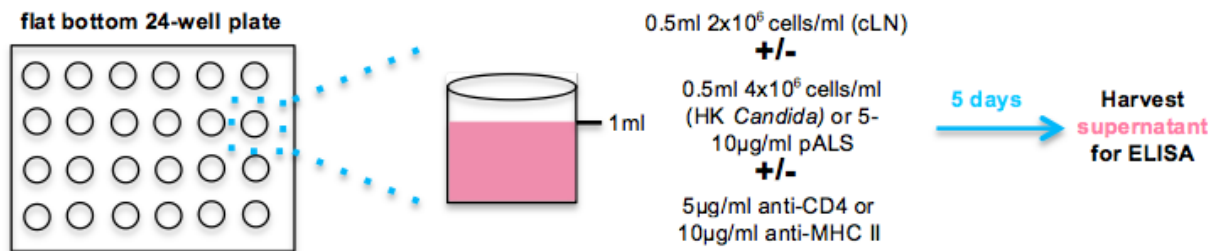
Figure 2.2: *In vitro* culture of cLN cells with HK *C. albicans*. Three to five days following re-challenge, cLN were harvested and homogenized to obtain single-cell suspensions. 1×10^6 cLN cells were plated and stimulated with 2×10^6 HK *C. albicans* or pALS +/- anti-CD4 or anti-MHC class II. 5 days after stimulation supernatants were collected and the concentration of IL-17 and IFNγ were measured by ELISA.

2.5   FLOW CYTOMETRY

One million cLN cells were rested overnight at 37°C and 5% CO_2 in serum-free AIM V media containing 1X β-Mercaptoethanol and 20U/ml of IL-2. Cells were subsequently stimulated with 50 ng/ml PMA and 500 ng/ml Ionomycin in the presence of Golgi Plug (BD Biosciences) for 4 hours at 37°C and 5% CO_2. Following stimulation, cells were stained for CD3 APC-Cy7 (clone 145-2C11), CD4 FITC (clone GK1.5), CD8 PerCP (clone 53-6.7), CD44 eFluor 450 (clone IM7), CD27 APC (clone LG.7F9), TCRβ (clone H57-597) and TCRδ (clone eBio GL3). IL-17 and IFNγ were detected after cell fixation and permeabilization according to the instructions of the Intracellular Staining Kit (BD Biosciences). Although in the majority of experiments cytokines were detected by intracellular cytokine staining (ICS), in certain occasions intracellular IL-17 and IFNγ were stained with Miltenyi Cytokine Secretion Assay (Miltenyi Biotec) according to the manufacturer's protocol. A comparison of both methods is depicted in **Figure 2.3.**
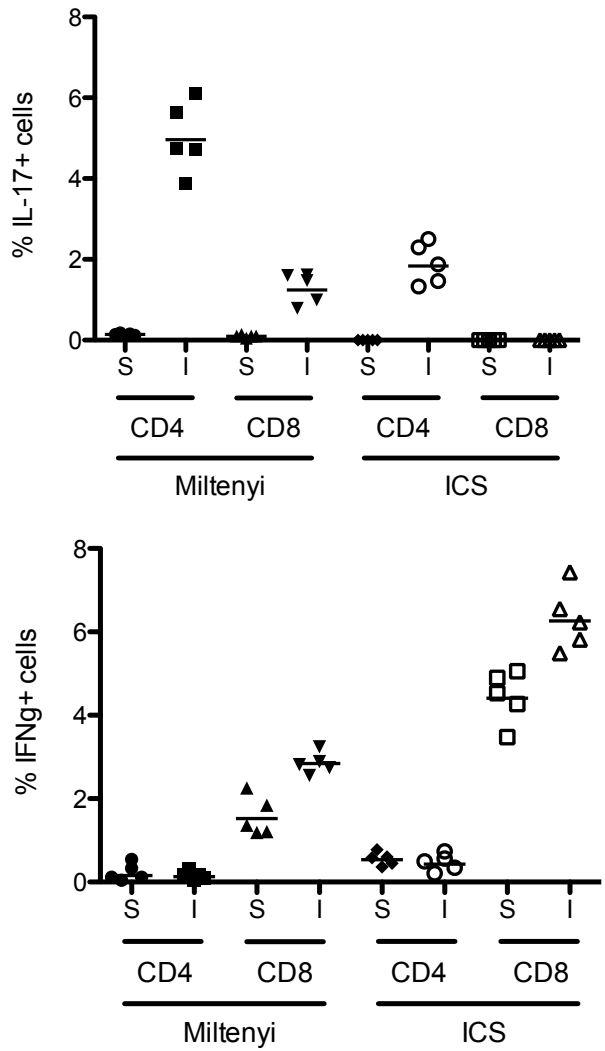
Figure 2.3: Comparison between ICS and Miltenyi Cytokine Secretion Assay.
Mice were re-challenged as in **Figure 2.1** and cLN were harvested for assessment of cytokine production by ICS and Miltenyi Cytokine Secretion Assay. The frequency of IL-17+ and IFNγ+ cells among CD4+ and CD8+ T cells is presented. Cells were gated on lymphocytes, CD3+ cells and CD4+ or CD8+ cells. Each point represents the data acquired from 1 mouse. The **S** indicates **Sham** and **I** indicates **Infected**.

2.6   DEPLETION OF CD4+ AND CD8+ T CELLS

WT, CD4-/- and CD8-/- mice were infected and re-challenged as described above **(Figure 2.1).** Depleting antibodies to CD4 and CD8, and the appropriate isotype controls, were administered i.p. at days -3 (200 µg) -2 (100 µg) and -1 (100 µg) relative to re-challenge (day 0) in 100 – 200 µl of sterile PBS **(Figure 2.4)**. Antibodies were received from BioXCell and reconstituted in sterile PBS at 1 mg/ml. Depletion of the appropriate T cell subsets was verified by flow cytometric analysis of CD4 and CD8 expression on the surface of splenocytes and cLN cells. This depletion protocol was kindly provided by Dr. Kyle C. McKenna from the Department of Ophthalmology at the University of Pittsburgh.
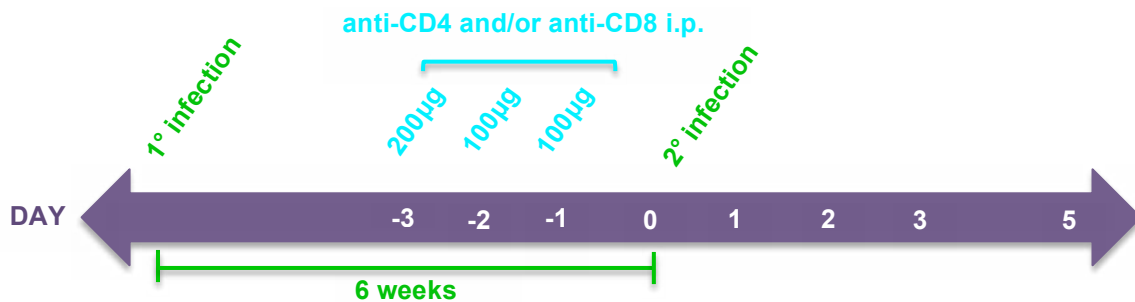
Figure 2.4: Scheme of CD4+ and CD8+ T cell depletion. CD4+ and CD8+ T cells were depleted alone or simultaneously at the indicated time points with 100-200µg of anti-CD4 and/or anti-CD8 antibodies. Depletion of the appropriate cell subsets was verified in spleen cell suspensions by flow cytometry.

2.7   ADOPTIVE TRANSFER

WT and CD4-/- mice were subjected to re-challenge **(Figure 2.1)** and cLN were subsequently harvested 3 days later. CD4+, CD8+ and CD4-CD8- (DN) cells were purified from the cLN by positive selection using magnetic beads (Miltenyi Biotec) according to the manufacturer's protocol. Ten million CD4+ and CD8+ cells or 2×10^7 DN cells were transferred i.p. into Rag1-/- mice in 200 20000 i.pr's protocol. Ten million CD4+ and CD8+*C. albicans.* Four days following primary infection, the tongue of Rag1-/- recipients was harvested for quantification of fungal burden and gene expression **(Figure 2.5)**.
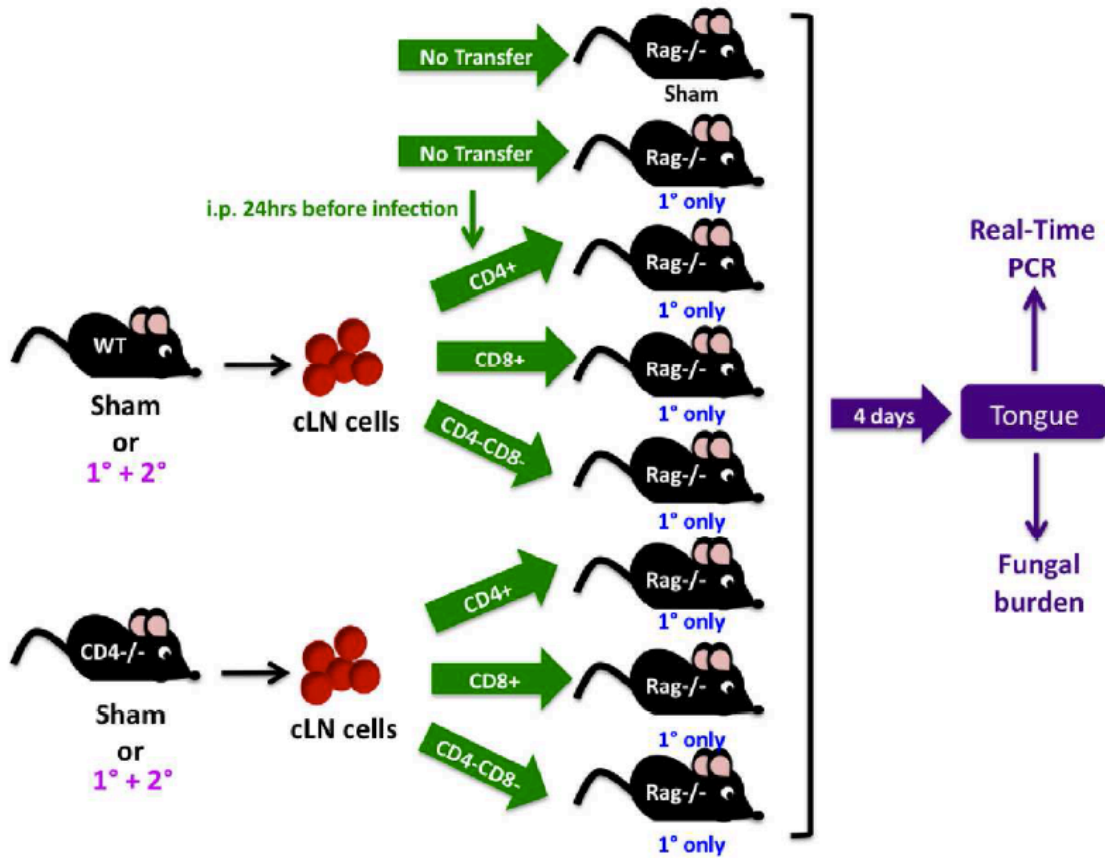
Figure 2.5: Adoptive transfer of CD4+, CD8+ and DN cells. WT and CD4-/-

mice were re-challenged and CD4+, CD8+ and DN cells were isolated from cLN

by magnetic sorting. Cell fractions were then transferred into Rag1-/- mice i.p. 1

day prior to infection with *C. albicans*. Four days after transfer tongue tissue

was harvested to enumerate *C. albicans* colonies and measure gene

expression.

2.8   REAL-TIME PCR

Frozen tongue tissue preserved in RNA later was homogenized in 500µl of RLT Lysis Buffer (RNAeasy Kit, Qiagen) using a GentleMACS Dissociator (M-tubes, RNA-02 program, Miltenyi Biotec). The lysates were then collected by centrifugation at 2000 rpm, 25°C for 2 minutes and RNA was subsequently extracted according to the instructions of the RNAeasy Kit. 1ently extracted according to the instrucDNA with the SuperScript III First-Strand Sythesis System (Invitrogen Carlsbad CA). Relative quantitation of gene expression was then performed with SYBR Green (Quanta BioSciences, Inc.) technology. GAPDH was used as a housekeeping gene to normalize threshold cycle (C_T) values. All primers, unless otherwise indicated, were from SA Biosciences (Qiagen Valencia, CA). TCRI primers were ordered from Eurofins MWG Operon and their sequence from 5'èromreads as follows TCRd forward **TAGTCACACAGGAGAGTTTTCC**, TCRd reverse **CTTTATTGTCTGCTTGGAGAGC**. Results were analyzed on a 7300 Real Time PCR System (Applied Biosystems Carlsbad, CA). mRNA expression was calculated using the 2^{-expr} method.

2.9   GENERATION OF OVALBUMIN-EXPRESSING CANDIDA

The strategy employed for the generation of *Candida*-OVA is depicted in **Figure 2.6**. OVA was fused to SSA2, a cell wall protein that interacts with Histatin 5, a component of human saliva with potent antifungal activity. First, the OVA sequence was optimized for codon usage in *C. albicans*, where CTG is decoded as serine instead of the

universal leucine (Massey et al., 2003). The optimized OVA sequence was then ligated to the 61bp sequence before the stop codon at the 5' end of SSA2, to allow for subsequent homologous recombination. The synthesis and optimization of the OVA sequence, and subsequent ligation to 61bp SSA2, was performed by GeneArt (Germany).   Second, the optimized sequence was inserted into a yeast vector containing the ADH1 transcriptional terminator and the orotidine-5'-monophosphate decarboxylase (URA3) gene, which is responsible for uridine synthesis and it was therefore used as a selection marker. Third, the 67bp sequence after the stop codon at the end of SSA2 was added by PCR at the 3' end of the recombination cassette. Primer sequence 5'èsynthesis and it was therefore used as a selection marker. Third, the 67bp sequence a after the stop codon at the end of SSA2 was added by PCR a Recombination cassette was amplified with TaKaRa Prime STAR HS DNA Polymerase (TAKARA BIO INC) **(Figure 2.6B)** under the cycling conditions depicted in **Table 2.1**. Finally, URA3 negative strain CAF4-2 was transformed with the recombination cassette **(Figure 2.6A)** and positive clones were selected in media (YNB) deprived of uridine. Yeast transformation was performed according to the instructions of the Zymo Research Frozen-EZ Yeast Transformation Kit II.   In order to verify integration of the recombination cassette into the genome, DNA was then extracted from URA+ transformants with Puregene Yeast/Bac. Kit B (Qiagen) and the junction region between the recombination cassette and the genomic SSA2 sequence was amplified **(Figure 2.6C)**.   PCR was carried out with TaKaRa Prime STAR HS DNA Polymerase and following the cycling conditions depicted in **Table 2.1** modifying the extension time to 1 minute. Primer sequences are depicted in **Table 2.2.** The clones that integrated the

recombination cassette into the genome were lysed for quantification of OVA protein expression.
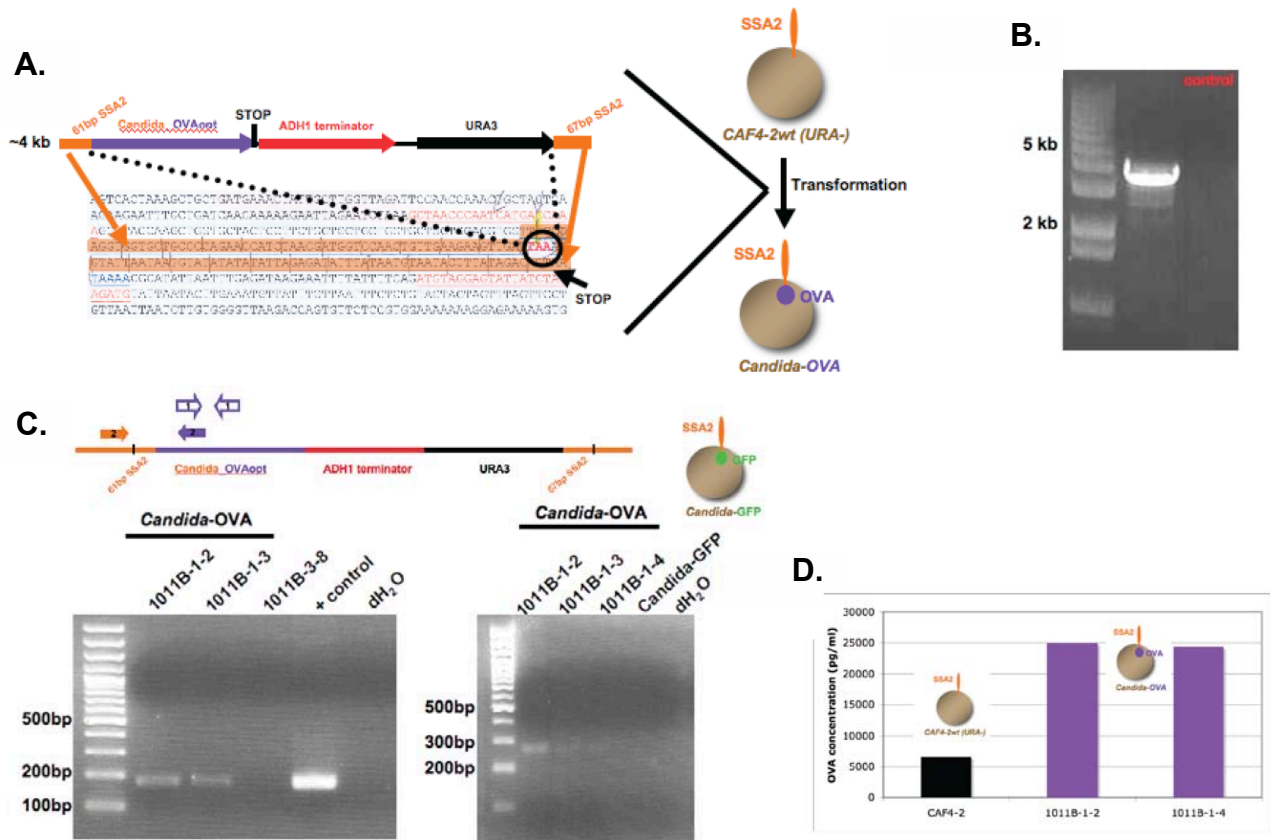
Figure 2.6: Generation of *Candida*-OVA. (A) Strategy employed in the generation of *Candida*-OVA. **(B)** PCR product after addition of 67bp of the SSA2 sequence at the 3' end of the recombination cassette. **(C)** Integration into the host genome was verified by amplification of a segment in the OVA sequence (primer pair 1 and left panel) or a sequence including the region upstream the recombination cassette and the OVA sequence (primer pair 2 and right panel). The positive control in the left panel is a plasmid containing the OVA sequence. The negative control in the right panel is *Candida*-GFP because GFP is located in the same position as OVA in *Candida*-OVA and therefore, should not be amplified. 1011B-1-2, 1-3, 1-4, and 3-8 represent individual clones. **(D)** OVA protein expression was verified by ELISA performed with yeast cell lysates generated by mechanical disruption with glass beads.

Table 2.1: PCR cycling conditions for amplification of recombination cassette with TaKaRa Prime STAR HS DNA Polymerase.

Step	Temperature (°C)	Time	
Initial denaturation	98°C	3 minutes	
Denaturation	98°C	10 seconds	
Annealing	55°C	5 seconds	30 cycles
Extension	72°C	4 minutes	
Final extension	72°C	10 minutes	

Table 2.2: Sequence of primers used to verify integration of OVA protein in the *Candida* genome.

Primer pair	Primer name	Primer sequence (5' to 3')
1 in Figure 2.6	SSA2_OVAopt_URA3F OVA_check_R_corre	CTGCCCCAGAACCATCTAAC AAACCATAGCCAAAGCTGACA
2 in Figure 2.6	SSA2_check F OVA_check_R_corre	GCTAACCCAATCATGACCAAA AAACCATAGCCAAAGCTGACA

2.10 YEAST CELL LYSATES

Yeast cell lysates were prepared by mechanical disruption with glass beads. 425-600 µe acid-washed glass beads (Sigma) were mixed with overnight yeast culture at a 1:1 ratio in lysis buffer (20mM Hepes pH 7.4, 150mM NaCl and Np40) containing protease inhibitor cocktail (Roche). Yeasts were then lysed via continuous cycles of vigorous agitation (vortex, 30 seconds) and cooling (ice, 30 seconds). Agitation and cooling cycle was repeated 8 times. The protein content in the lysates was measured using the Bradford technique and the Bio-Rad Protein Assay. Lysates were then aliquoted and stored at -80°C.

2.11 OVALBUMIN ELISA

Ovalbumin expression was verified by ELISA. A 96 well plate was coated overnight at 4°C with 100h   per well of **goat IgG fraction to chicken egg albumin** (MP Biomedicals) diluted 1:1000 in PBS. Plate was then washed 3 times with 200Pl/well of PBS/Tween 20 (diluted 1:2000). 100nSA. A 96 well plasate was added to each well and incubated for 2 hours at room temperature. The lysate was subsequently aspirated and the plate washed 5 times with PBS/Tween and once with PBS. Non-specific binding was blocked with 100µl/well of 5% milk for 1 hour at room temperature. Milk was removed by washing 2 times with PBS/Tween. 100µo/well of polyclonal **rabbit IgG fraction to chicken egg albumin** (MP Biomedicals) diluted 1:100,000 in PBS were added and incubated at room temperature for 2 hours. Primary antibody excess was

removed by washing 5 times with PBS/Tween. To allow detection of ovalbumin-bound primary antibody, **horseradish peroxidase-conjugated donkey anti-rabbit IgG** was diluted in PBS 1:2500 (Jackson Immuno Research Laboratories) and 100e /well were added and subsequently incubated at room temperature for 1 hour. The excess of enzyme-conjugated antibody was removed by washing with PBS/Tween 2 times and with PBS 3 times. 100Th/well of horseradish peroxidase substrate (TMB Stabilized Chromogen, Invitrogen) were added and incubated for 30 minutes at room temperature and protected from light. The colorimetric enzymatic solution was terminated with stop solution (Invitrogen) and absorbance was measure at 450nm wavelength with a 540nm correction. ELISA protocol was adapted from the instructions kindly provided by Dr. Robert Binder from the Department of Immunology at the University of Pittsburgh.

2.12   CFSE LABELING AND PROLIFERATION ASSAY

Pooled lymph node and spleen cells from OTII transgenic mice were labeled with 1 μo Carboxyfluorescein diacetate Succinimidyl Ester (CFSE) (Molecular Probes, Invitrogen) by mixing the cells with CFSE (described in **Figure 2.7)** diluted in PBS to the appropriate concentration followed by a 15 minutes incubation period at 37°C**.** To remove the unbound CFSE, cold FBS was added to the CFSE-labeled cell suspension at a 1:1 ratio and incubated on ice for 5 minutes. Cells were then pelleted (1,500 rpm, 4°C, 5 minutes) and washed once in complete RPMI1640 containing 10% FBS, L-glutamine and antibiotics (Penicillin and Streptomycin). Subsequently, 10ml of pre-warmed, complete RPMI 1640 were added to the cells followed by a 30 minute

incubation at 37°C. Cells were then collected by centrifugation (1,500 rpm, room temperature, 5 minutes) and resuspended in complete RPMI 1640 at a density of 2 x 10^6 cells/ml. 100ls/ml. of the CFSE-labeled cells were plated in round-bottom 96-well plates and subsequently stimulated with ~1.5 x 10^6 HK *Candida*-OVA cells or the parental URA- strain CAF4-2. HK *Candida* was prepared as described in **section 2.4**. As positive and negative controls cells were stimulated with 20 As p of $OVA_{323-339}$ (AnaSpec) or left without stimulus, respectively. CFSE-labeled OTII cells were incubated in the presence of stimulus for 5 days at 37°C and 5% CO_2. We then proceeded to analyze the cells by flow cytometry.
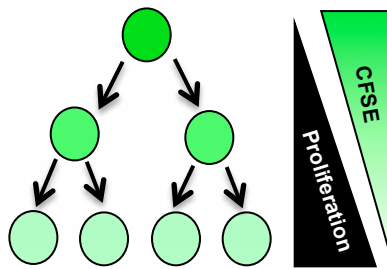
Figure 2.7: Carboxyfluorescein diacetate succinimidyl ester (CFSE) mechanism of action. CFSE is colorless until it enters the intracellular space where its acetate groups are cleaved by intracellular esterases yielding a highly fluorescent green dye. The succinimydil group reacts with intracellular amines, forming stable CFSE-protein complexes. The remaining unbound dye diffuses outside the cell, where it can be washed away. Due to the binding of CFSE to intracellular proteins, it is diluted 2 fold with every cell division as the total protein content of the cell splits in half. This makes CFSE a powerful tool to track cellular proliferation as the concentration of dye decreases.

In one occasion the proliferation assay was performed with purified CD11c+ cells and CFSE-labeled CD4+ T cells. In this case, OTII spleens and LN were perfused or bathed with a 2mg/ml solution of collagenase D and incubated at 37°C for 30 minutes. The tissue was subsequently homogenized by mechanical disruption. In order to acquire enough numbers of total cells the lymph nodes and spleens of 3 OTII mice were pooled. CD4+ and CD11c+ cells were then purified by magnetic sorting using negative and positive selection, respectively. Labeling with magnetic beads and subsequent sorting were performed according to the manufacturer's instructions (Miltenyi Biotec). $1x10^5$ CD11c+ and CFSE-labeled CD4+ T cells were plated and stimulated with $1x10^6$ HK *Candida* and incubated for 5 days, which was followed by flow cytometric analysis.

In a separate experiment we tested the proliferation of OTII cells upon stimulation with live *Candida*-OVA. 2×10^5 live *Candida*-OVA cells were added to 2×10^5 CFSE-labeled total spleen and lymph node cells in the presence of 5µg/ml of fluconazole in order to avoid yeast overgrowth. The fluconazole was added after 2 hours of incubation at 37°C and left in the culture media for the entire 5-day incubation period.

2.13   HISTOLOGY

Tongue tissue was preserved in 10% NBF, parafilm embedded, sectioned and stained with hematoxylin and eosin (H&E) and periodic acid schiff (PAS). In order to observe neutrophils and *C. albicans* in the same lesions, serial sections were obtained and one section was stained with H&E and the following section with PAS. Tissue sectioning and staining were performed by the Research Histology Services located in the 15th floor of the Thomas E. Starzl Biomedical Science Tower.

2.14   STATISTICS

Unless otherwise indicated, all data was analyzed with a two-tailed unpaired *t* test using the Wilcoxon Mann-Whitney correction. Statistically significant differences are indicated by $*p<0.05$.

3.0   CHAPTER 3: *C. ALBICANS* RE-CHALLENGE INDUCES LONG-TERM

PROTECTIVE TH17 IMMUNITY

3.1 BACKGROUND

In recent years accumulating evidence from clinical studies has demonstrated an undeniable association between CMC and genetic defects in the IL-17/Th17 pathway **(Figure 1.2 and Table 1.1)**, indicating Th17-mediated responses are crucial in host defense against *C. albicans.* In addition, we have observed that *C. albicans* persists in IL-23-/- mice 21 days after inoculation **(Figure 3.1)** suggesting that in the absence of IL-23, which is instrumental for the maintenance of Th17 cells, *C. albicans* establishes chronic infection. Moreover, Rag1-/- mice, which lack functional B and T cells, are susceptible to OPC (Pandiyan et al., 2011). These data strongly suggest a role for "classical" Th17 cells in host defense to OPC.

However, IL-17 production is not an exclusive function of Th17 cells; several innate sources have been described and are summarized in **Table 3.1** (Cua and Tato, 2010). For example, γδ T cells are potent IL-17 producers at mucosal surfaces. Interestingly, they express dectin-1 (Martin et al., 2009), which is important for the recognition of β-glucan, a component of the *C. albicans* cell wall. It is important to consider that the anatomy and physiology of the immune system in the oral mucosa has not been described in detail, although there is evidence from human studies suggesting the existence of oral immune foci (Cutler and Jotwani, 2006). It is not known if these innate sources of IL-17 are present in the tongue at steady state and whether they play a role in response to *C. albicans* infection. In support of the notion of innate IL-17 sources during OPC are the findings that both IL-17 **(Figure 3.2)** and its target genes (CXCL1, CXCL5, S100A8, etc.) were detected in the tongue as early as 24 hours

following infection (Conti et al., 2009), suggesting that an innate source(s) of IL-17 plays an important role in host defense during OPC.

In light of these data, the goal of the experiments outlined in this chapter was to define the role of "classic" Th17 cells in immunity to *C. albicans* relative to innate responses during OPC.

Table 3-1: Innate sources of IL-17

Cell type	Location	Function	References
γδ T cell	Gut and skin	Surveillance; rapid host defense; maintenance of barrier function	(Hayday, 2009; Lochner et al., 2008; Martin et al., 2009; Sutton et al., 2009)
iNKT cell	Liver, lung and skin	Surveillance; rapid host defense	(Doisne et al., 2009; Hayday, 2009; Michel et al., 2008)
CD3-NKp46+	Gut and skin	Production of pro-inflammatory mediators	(Cella et al., 2009; Crellin et al., 2010; Luci et al., 2009; Sanos and Diefenbach, 2010; Satoh-Takayama et al., 2010; Satoh-Takayama et al., 2008)
LTi-like cell	Lamina propria and spleen	Surveillance; lymphoid aggregate formation?	(Buonocore et al., 2010; Colonna, 2009; Cupedo et al., 2009; Takatori et al., 2009)
THY1+SCA1+CD3– CD4–KIT– cell	Lamina propria	Immune surveillance	(Buonocore et al., 2010)
Paneth cell	Intestinal crypts	Surveillance; amplification of immune response	(Takahashi et al., 2008)
GR1+CD11b+ cell (Neutrophils)	Lung and kidney	Host defense	(Ferretti et al., 2003; Li et al., 2010; Werner et al., 2011)

Adapted from Daniel J. Cua and Cristina M. Tato, Nature Reviews Immunology, 2010
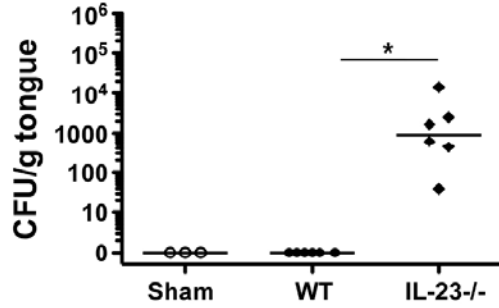
Figure 3.1: *C. albicans* establishes persistent infection in IL-23-/- mice.

Mice were inoculated with *C. albicans* and the tongue tissue was harvested for

colony enumeration 21 days following infection.
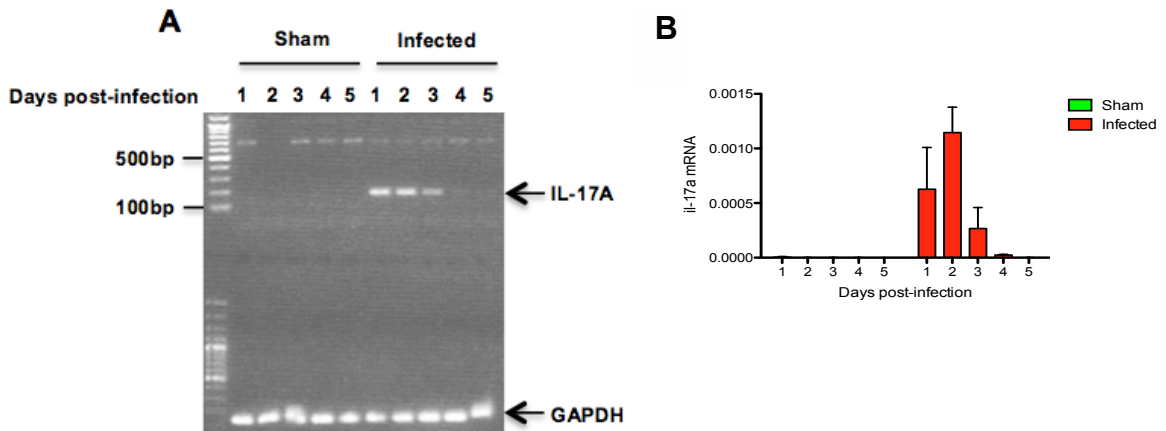
Figure 3.2: IL-17 expression in tongue tissue following primary infection.

WT mice were sham or *C. albicans* infected and the tongue tissue was harvested

at days 1-5 post-infection for subsequent RNA extraction and PCR. IL-17A was

amplified from tongue cDNA via traditional PCR **(A)** or real-time PCR **(B).** IL-

17A-specific amplification was verified via the dissociation curve method, which

yielded a single peak.

3.2   RESULTS

3.2.1 Pre-exposure to *C. albicans* results in accelerated fungal clearance.

In order to study the specific contribution of adaptive immune responses to fungal clearance, we developed a recall model of infection **(Figure 2.1).** Since *C. albicans* is not a commensal microbe in mice, this approach also allowed us to better model the human condition, where *C. albicans* is a component of the normal flora in mucosal surfaces. Experimental cohorts were designed to compare tongue fungal burdens between mice that received 1° infection alone or both 1° and 2° challenge. We hypothesized that upon pre-exposure to *C. albicans* we would observe accelerated fungal clearance due to the development of long-term protective immunity. As expected, mice that received 1° and 2° infections experienced accelerate fungal clearance at days 1 and 2 post re-challenge, as demonstrated by a ~1 log decrease in tongue fungal burden (**Figure 3.3**). Infection was cleared by day 3 regardless of whether or not mice were pre-exposed to *C. albicans.* These data indicated that adaptive immune responses contribute to fungal clearance in OPC.

3.2.2 Accelerated fungal clearance correlates with increased frequency of CD4+IL-17+ (Th17) but not CD4+IFNγ (Th1) cells.

The important contribution of adaptive immune responses to *C. albicans* in OPC prompted us to study in detail the nature of the CD4+ T helper cell response responsible

for fungal clearance. To this end, mice were given 1° and 2° infection and cLN were subsequently harvested at days 1, 2 and 3 post-re-challenge. CD4+IL-17+ (Th17) cells were first detected 3 days after re-challenge in both mice that received 1° infection alone or followed by 2° challenge **(Figure 3.4A)**. Importantly, the frequency of Th17 cells was significantly increased in mice subjected to re-challenge when compared to mice that solely received 1° infection **(Figure 3.4B)**, indicating the expansion of Th17 cells upon a second encounter with *C. albicans.* We did not detect CD4+IFNγ+ (Th1) cells at any time point following re-challenge. Therefore Th17, but not Th1, cells are induced upon *C. albicans* re-challenge and are associated with decreased fungal clearance.

3.2.3 Th17 cells induced upon re-challenge exhibit an activated phenotype

A recent study in memory CD4 T cells induced after *Lysteria monocytogenes* infection showed that Th17 cells lacked expression of CD27, which has been shown in memory CD8+ T cells to be associated with a short life span. These observations suggested that memory Th17 cells are short-lived. Furthermore, adoptive transfer of CD4+**CD27-**CD44+ or CD4+**CD27+**CD44+ cells from *L. monocytogenes*-infected mice into naïve hosts resulted in 80% decrease of CD4+**CD27-**CD44+ cells whereas CD4+**CD27+**CD44+ cells survived 14 days after transfer (Pepper et al., 2010). However, a different study showed that, in spite of their lack of CD27 expression, Th17 cells were long-lived, exhibited "stem-cell-like" features and were functional against tumors (Muranski et al., 2011). Regardless of the differences in the longevity of Th17

cells, which is likely due to variations in the immunological milieu where they exert effector functions, Th17 cells appear to be CD27 negative.

Thus, in order to gain insight into the phenotype of *C. albicans*-specific Th17 cells induced upon re-challenge we examined expression of CD27 in combination with CD44, which is an indicator of T cell activation. We observed that the majority of *C. albicans*-specific Th17 cells were CD27-CD44+ **(Figure 3.5)**, suggesting an activated, effector phenotype.

3.2.4   CD4+ T cells are the primary source of IL-17 during recall responses and are antigen-specific.

CD4+ T cells are not the only possible source of IL-17 in the lymph node microenvironment. It has been demonstrated that CD8+ T cells can produce IL-17 (Tc17) and are induced upon influenza and vaccinia virus infections, although their cytotoxic activity is debated (Yeh et al., 2010; Zou et al., 2011). Therefore, we examined IL-17 production by CD4+ and CD8+ T cells in cLN following re-challenge. CD4+, but not CD8+, T cells produced IL-17 upon 2° infection **(Figure 3.6A-B)**, indicating that CD4+ T cells are the primary source of Il-17 during re-challenge. We also detected an infection-dependent increase of IFNγ production in the CD8+ T cell population but it appeared not to be a stable phenotype since it was observed only at day 3 post re-challenge and was completely undetectable by day 5 **(Figure 3.6B)**.

The facts that CD4+IL-17+ cells are detectable only after re-challenge and are completely absent in mice that received a sham infection suggested that these Th17 cells were antigen-specific. In order to explore this in more detail, we re-stimulated cLN

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cells *in vitro* with heat killed (HK) *C. albicans* or an ALS peptide (pALS), which is a sequence of the *C. albicans* ALS1/3 adhesins and was recently shown to be a CD4+ T cell epitope (Bar et al., 2012). We also included *C. albicans* strain CJN 1348 (Nobile et al., 2008), which lack the ALS1/3 proteins (depicted as ALS-/-), and the appropriate isogenic WT strain (CAYC2QTPU1, depicted as ALS+/+). In addition, to determine if these responses were CD4 and MHC II- dependent, we stimulated the cells in the presence or absence of blocking antibodies (Bar et al., 2012; Scurlock et al., 2011). The data showed that cLN cells produced IL-17 in a CD4- and MHC class II-dependent manner in response to WT HK *C. albicans* and the pALS peptide. The cells did not produce IL-17 in the absence of stimulus. Interestingly, stimulation with ALS1/3-deficient strain CJN 1348 resulted in IL-17 levels comparable to those resulting from stimulation with WT *C. albicans,* indicating the existence of other CD4+ T cell epitopes that stimulate IL-17 production **(Figure 3.7A)**. In addition, CD4- and MHC II-dependent production of IFNγ upon *in-vitro* re-stimulation was observed **(Figure 3.7B)**, which contrasts with the flow cytometry data where Th1 cells were not detected. However, the IFNγ levels detected in the *in vitro* co-cultures are modest (~10-100 pg/ml) compared to the large amounts of IL-17 (~500-2000 pg/ml) measured **(Figure 3.7)**. Therefore, *C. albicans* re-challenge induced primarily CD4+IL-17+ cells in an antigen-specific manner.

The study of antigen-specific responses in *C. albicans* infection models has been hampered by the lack of reagents such as TCR-specific transgenic mice. To address *C. albicans*-specific T cell responses *in vivo* during OPC, we created a strain of *C. albicans* that expresses chicken ovalbumin (*Candida*-OVA) in order to track T cell responses using the OTII transgenic system, in which all CD4 T cells express a TCR specific for

OVA peptide 323-339 (OVA$_{323-339}$). **Figure 2.6C-D** shows that two of the recombinant yeast clones we generated integrated the OVA sequence into the genome and expressed OVA protein as measured by ELISA. Next, it was important to test whether recombinant *Candida*-OVA was recognized by OTII cells *in vitro* using lymphocyte proliferation as readout. Lymphocyte proliferation was measured by monitoring dilution of CFSE, a dye that binds to intracellular proteins and dilutes with every cell division **(Figure 2.7).** Disappointingly, we observed no proliferation of OTII cells upon *Candida*-OVA stimulation, whereas lymphocytes proliferated significantly in the presence of OVA$_{323-339}$ **(Figure 3.8A)**. In order to ensure that the absence of proliferation of OTII cells was not due to denaturation of the OVA protein as a result of the heat killing process, we cultured OTII cells in the presence of live *Candida*-OVA, but no proliferation was observed **(Figure 3.8B).**

Taken together, these observations indicate that *C. albicans* re-challenge primarily induces IL-17 in a CD4 and antigen-dependent manner. However, tracking *C. albicans*-specific responses *in vivo* was not possible due to the fact that our *Candida*-OVA did not stimulate *in vitro* proliferation of OTII splenocytes.

3.2.5 CD4+ T cells induced upon re-challenge protect Rag1-/- mice from OPC.

Thus far, we have demonstrated that adaptive immune responses play an important role in fungal clearance, presumably due to the induction of Th17 cells upon re-challenge. However, these data did not prove that these Th17 cells are directly responsible for fungal clearance. Therefore, we isolated CD4+ T cells from total cLN cells of WT mice 3 days post 2° infection and adoptively transferred them into Rag1-/- host one day prior

to infection. We observed that Rag1-/- mice that received CD4+ T cells were protected from OPC whereas mice receiving CD4- cells were not protected **(Figure 3.9A, left panel)**. We also tracked weight loss post-infection since it is usually a good indicator of disease progression. However, we detected no significant differences between mice that received CD4+ T cells and mice that received CD4- or no cells. **(Figure 3.9A, right panel)**. Importantly, the homing of donor CD4+ T cells into the tongue of Rag1-/- mice correlated with increased expression of IL-17 as measured by real-time RT-PCR of tongue tissue, suggesting that Th17 cells are recruited to the tongue during infection and contribute to fungal clearance **(Figure 3.9B)**. Importantly, we detected no IFNγ following transfer of *Candida*-primed cells, confirming our finding that *C. albicans*-specific CD4+ T cell responses are primarily Th17.
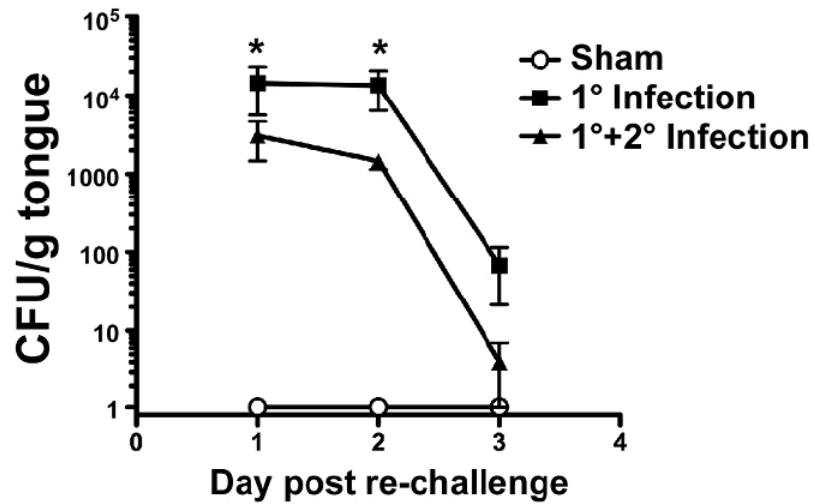
Figure 3.3: Pre-exposure to *C. albicans* results in accelerated fungal clearance. Mice were subjected to 1° infection alone or followed by 2° challenge (1°+2°). As a negative control mice received 2 consecutive sham infections. Tongue tissue was harvested and plated for colony enumeration at days 1-3 post re-challenge. Experiment was repeated twice and the pooled data from both experiments is depicted.
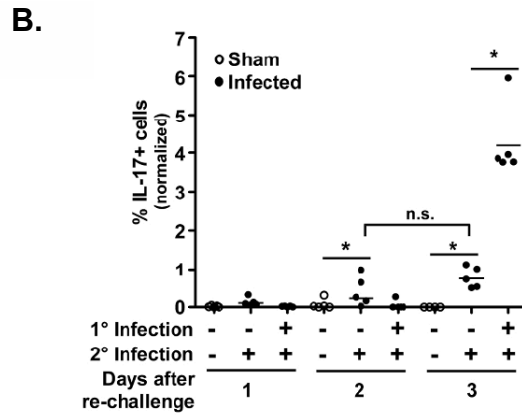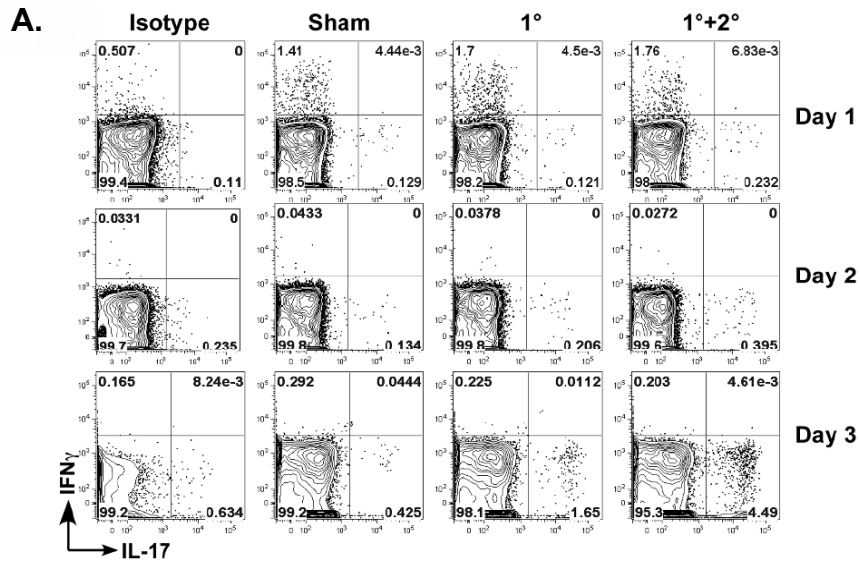
Figure 3.4: *C. albicans* re-challenge induces Th17 but not Th1 cells. **(A)** cLN cells from the experiment described in **Figure 3.3** were isolated 1-3 days after re-challenge and stained for CD4, IL-17 and IFNγ. Lymphocytes were gated on CD4+, and IL-17+ and IFNγ+ cell frequencies are depicted. **(B)** Summary of CD4+IL-17+ cell frequencies (from panel **A**) normalized to the isotype control.
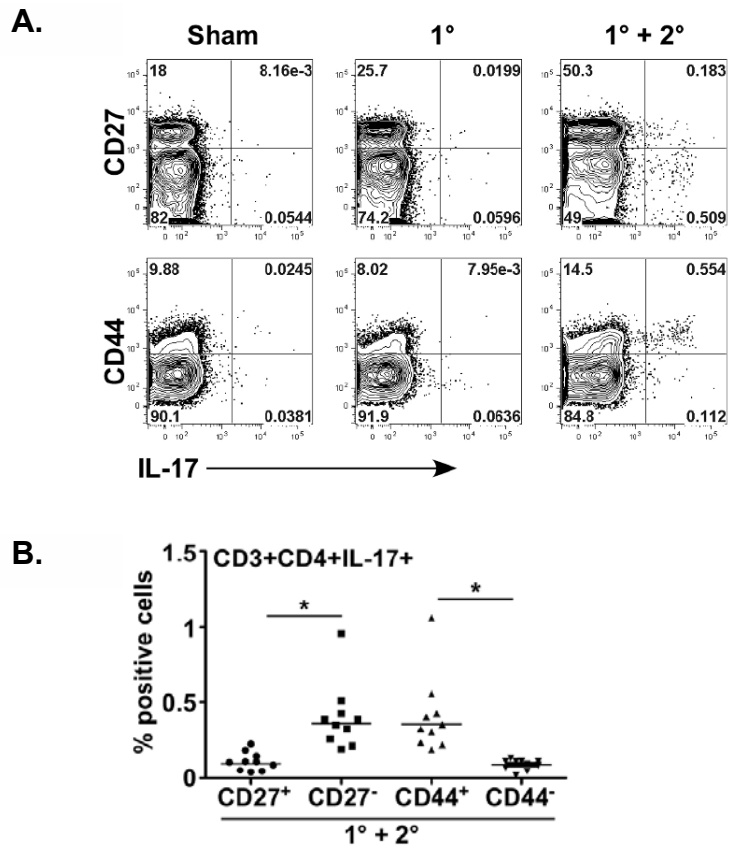
Figure 3.5: CD27 and CD44 expression on the surface of Th17 cells induced upon re-challenge. cLN cells were isolated 3 days after 1° infection or re-challenge and analyzed by flow cytometry for surface expression of CD27 and CD44 and intracellular IL-17. Lymphocytes were gated on CD3 and CD4 and the frequencies of IL-17+CD27+, IL-17+CD27-, IL-17+CD44+ and IL-17+CD44- cells are depicted as representative FACS plots **(A)** and as a summary of all the data collected **(B).**
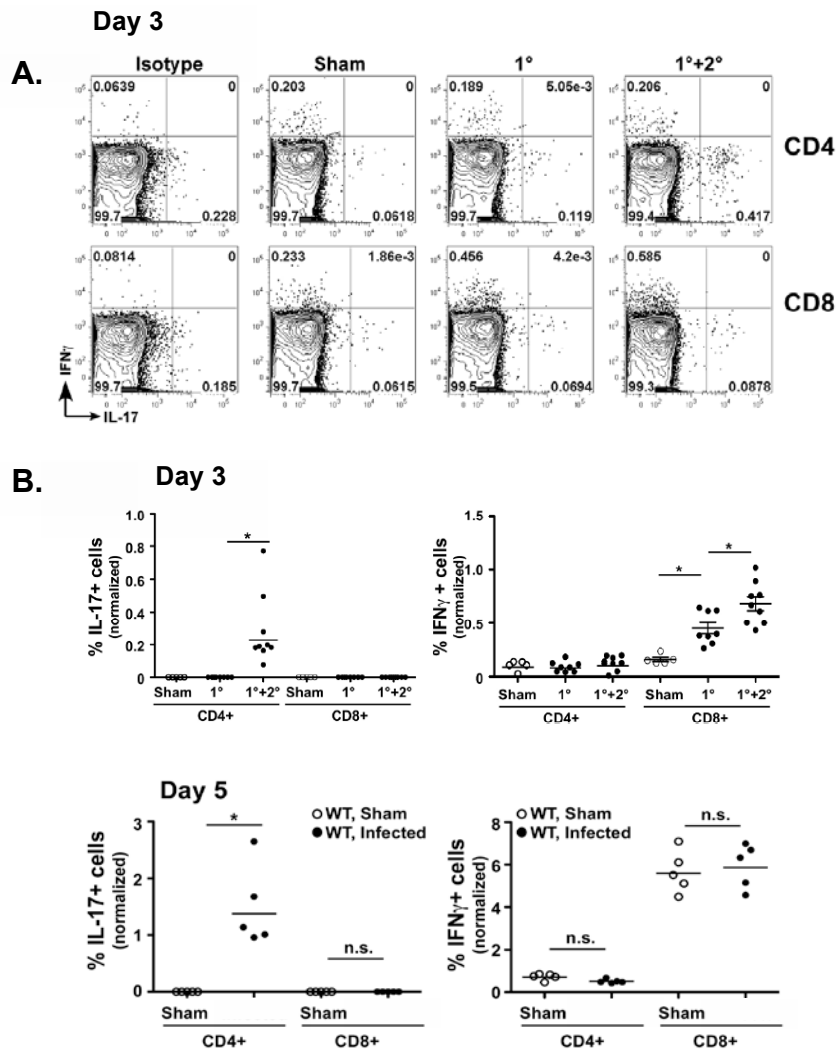
Figure 3.6: CD4+ T cells are the primary source of IL-17 during re-challenge. cLN cells were isolated 3 or 5 days after re-challenge and subsequently analyzed by flow cytometry. Lymphocytes were gated on CD3 and CD4 or CD8 and the frequency of IL-17+ and IFNγ+ cells is displayed in panel **A** for one representative mouse and summarized in **B** for all the mice included in this experiment.
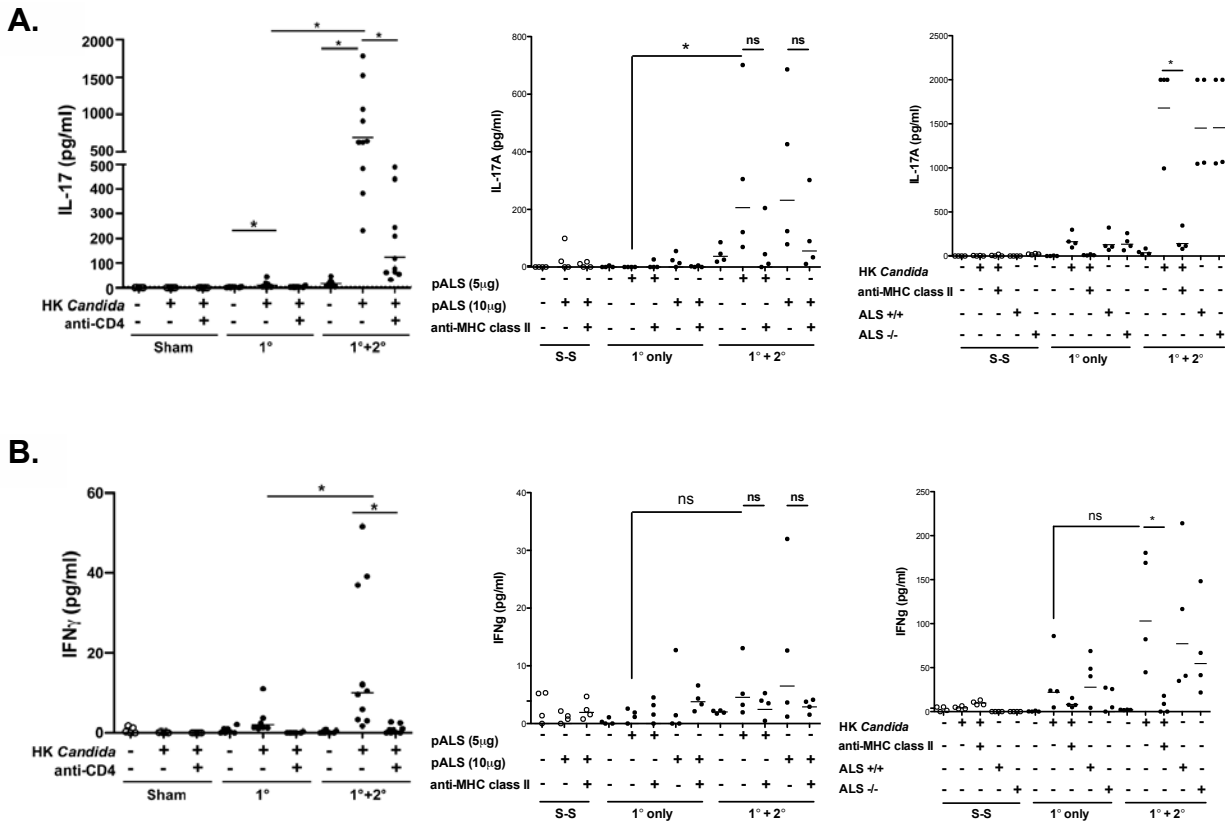
Figure 3.7: Th17 cells induced upon re-challenge are antigen-specific. WT mice were subjected to sham, 1° only or 1° + 2° infections and 3 days post re-challenge 1 x10^6 cLN cells were re-stimulated *in vitro* with 2 x 10^6 HK *C. albicans* or pALS at the specified concentrations in the presence or absence of anti-CD4 (5μg/ml) or anti-MHC class II (10μg/ml) blocking antibodies. The strains of *C. albicans* used were WT CAF 2-1 (depicted as *Candida*), CAYC2QTPU1 (depicted as ALS+/+) and CJN 1348 (depicted as ALS-/-). 5-4 days after *in vitro* stimulation, the supernatants were harvested and the concentration of IL-17 **(A)** and IFNγ **(B)** was measured by ELISA.
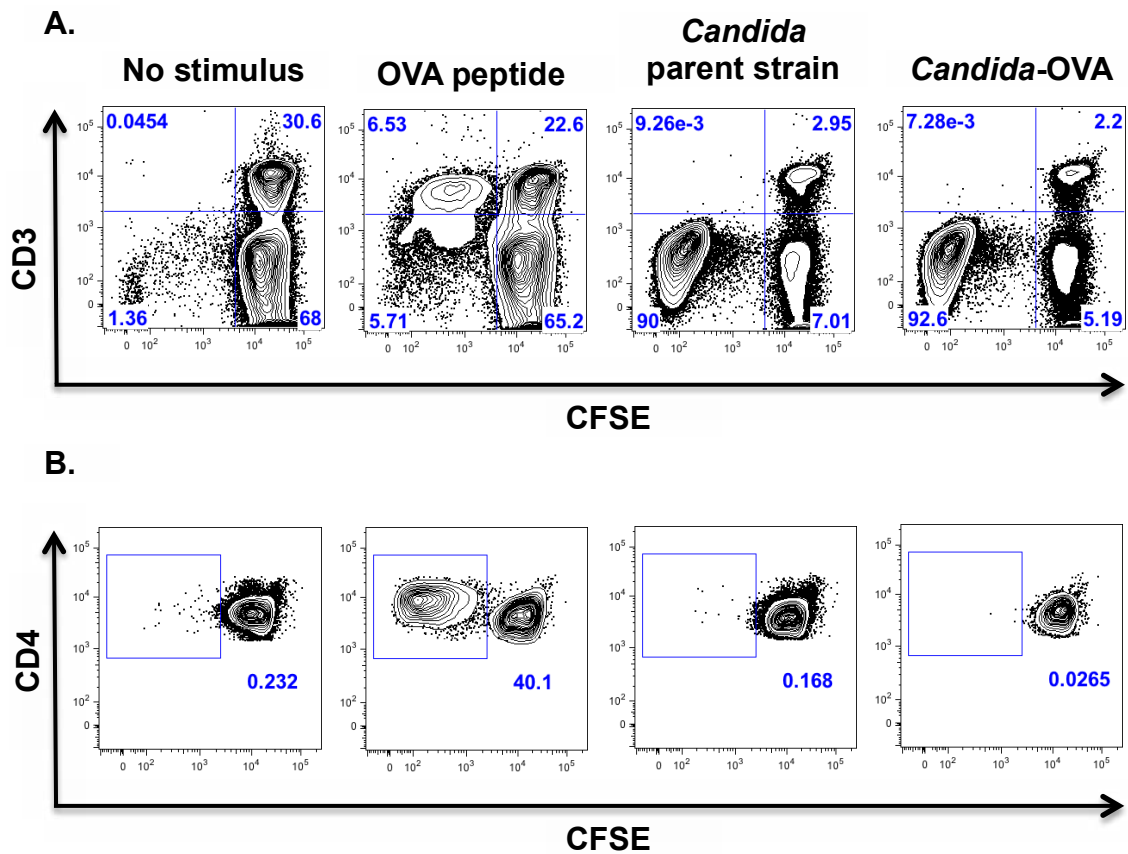
Figure 3.8: ***Candida*-OVA does not stimulate proliferation of OTII splenocytes *in-vitro*.** **(A)** OTII splenocytes were labeled with CFSE and incubated for 5 days in the absence of stimulus or in the presence of OVA peptide (OVA$_{323-339}$), the parental HK *C. albicans* strain (no OVA) or HK *Candida*-OVA.. Following incubation the cells were harvested and analyzed for CFSE dilution. Cells were gated on lymphocytes and CD3 vs. CFSE is depicted. **(B)** Experiment was performed as in **(A)** with the exception that OTII splenocytes were incubated with live *C. albicans.* In order to avoid *C. albicans* overgrowth fluconazole was added. Lymphocytes were gated on CD3+CD4+ cells and CD4 vs. CFSE is displayed.
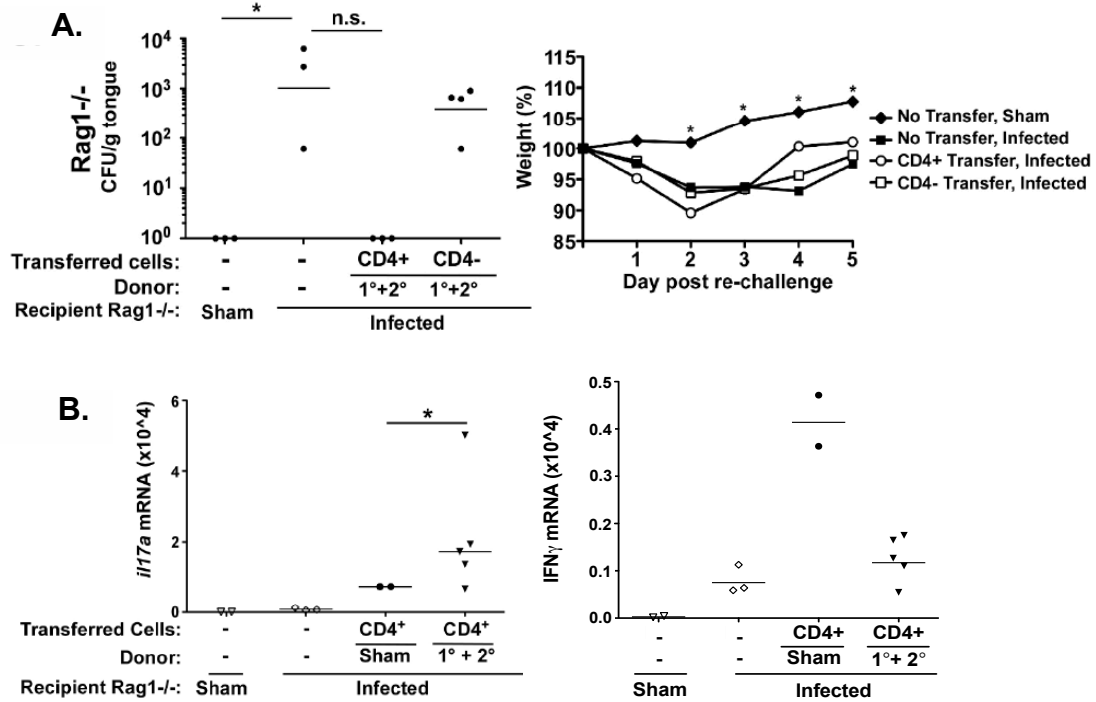
Figure 3.9: CD4+ T cells induced during re-challenge protect Rag1-/- mice from OPC and their homing into the tongue associates with increased IL-17A expression. WT mice were subjected to sham or 1° and 2° infections and cLN were harvested 3 days following re-challenge. CD4+ and CD4- cells were purified by magnetic sorting (positive selection) from cLN cells and transferred i.p. into Rag1-/- mice 24 hours prior to infection. As controls for the infection, Rag1-/- that received no cells (PBS) were either sham- or *C. albicans*-infected. Five days after infection the tongue of Rag1-/- mice was harvested and homogenized for both colony enumeration **(A, left panel)** and relative quantitation of IL-17A and IFNγ expression by real-time PCR **(B).** The weight of Rag1-/- mice was monitored every day for the duration of the experiment and it is shown as percent of the original weight **(A, right panel)**. Panels A and B resulted from 3 separate experiments.

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3.3   DISCUSSION

To the best of our knowledge, this is the first study where the relative role of adaptive and innate immune responses to OPC had been addressed.   Furthermore, we demonstrated that pre-exposure to *C. albicans* resulted in accelerated fungal clearance, which was associated with the development of long-term *C. albicans*-specific Th17 responses.   Even more importantly, Th17 cells induced upon re-challenge protected Rag1-/- mice from OPC.   Thus, Th17 cells are an important source of IL-17 during re-challenge and contribute to fungal clearance.

Upon pre-exposure to *C. albicans,* we achieved approximately a 1 log decrease in fungal burden, which is a significant finding considering that experimental vaccines to *C. albicans* confer, at most, approximately a 2 log protection.   In fact, the only experimental vaccine (recombinant N-terminus of Als3) which efficacy was tested in OPC provided less than 1 log protection upon challenge with *C. albicans* (Spellberg et al., 2006).   During disseminated candidiasis, administration of a vaccine that consisted of DC transfected with *C. albicans* RNA resulted in a 5-fold decrease in fungal burden when compared to controls (Bozza et al., 2004).   In addition, vaccination with antibody-inducing preparations of *C. albicans* cell wall proteins and carbohydrates resulted in, at most, a ~2 log decrease in fungal burden following intravenous or intravaginal challenge (Casadevall and Pirofski, 2012).   In addition, there are no fungal vaccines currently used for treatment.   Therefore, it is difficult to define a quantitatively significant protection with no point of reference.

The accelerated fungal clearance we observed upon pre-exposure to *Candida* was associated with an increase in the frequency of antigen-specific CD4+IL-17+ T cells

in the cLN. My attempt to perform a more detailed study of antigen-specific Th17 responses was hampered by the observation that *Candida*-OVA did not stimulate proliferation of OTII splenocytes *in vitro.* We are not certain of what caused this phenomenon. One possibility is that since OVA was fused to SSA2, a membrane protein, it is not secreted. This could limit the accessibility of APCs to OVA protein and thus the OVA$_{323-339}$ peptide responsible for the activation of OTII cells. In fact, initial studies using DO11.10 TCR transgenic T cells (also specific for OVA$_{323-339}$) to track *in vivo* T cell responses employed *E. coli*-OVA, where OVA was fused to a secreted protein (Chen and Jenkins, 1998). However, in a recent elegant study others engineered a *C. albicans* strain where OVA$_{323-339}$ epitope, and other model antigens, were fused in frame to the enolase gene (Calb-Ag), which does not encode a secreted protein (Igyarto et al., 2011). Of note, here they fused the epitope directly, not the whole OVA protein, which may have increased the accessibility of APCs to the antigen. In fact, Calb-Ag was able to stimulate proliferation of OTII T cells *in vitro* and, upon antigen processing and presentation by Langerhan's Cells in the skin, induced Th17 development.

In spite of the technical difficulties experienced while studying antigen-specific T cell responses in OPC, we demonstrated that *C. albicans* induced CD27-CD44+ Th17 cells upon re-challenge. Furthermore, re-challenge-induced CD4+ T cells protected Rag1-/- mice from OPC, which correlated with a local increase in IL-17 expression upon homing of CD4+ T cells to the tongue of Rag1-/- mice. Therefore, we believe that when Th17 cells encounter *C. albicans* during re-challenge, they migrate to the tongue and cooperate with local innate IL-17 sources to clear *C. albicans.*

4.0 CHAPTER 4: COMPENSATORY SOURCES OF IL-17 DURING *C. ALBICANS* RE-CHALLENGE IN CD4-DEFICIENT MICE

4.1   BACKGROUND

In chapter three it was demonstrated that adaptive immune responses contributed to *C. albicans* clearance from the oral cavity and that fungal eradication was associated with an increased frequency of antigen-specific Th17 cells, suggesting that Th17 cells are essential for fungal clearance. This is in accordance with three important observations in humans: (1) the majority of *C. albicans*-specific T cells in peripheral blood exhibit a classic Th17 phenotype (CD4+CCR4+CCR6+IL-17+) (Acosta-Rodriguez et al., 2007), genetic mutations at various stages of the Th17 pathway **(Figure 1.2 and Table 1.1)** are associated with the development of CMC, (3) Approximately 95% of HIV+ individuals develop overt "thrush" lesions and thus, OPC is considered an AIDS defining illness (Akpan and Morgan, 2002). In light of these observations, it was important to determine whether Th17 cells were absolutely required for fungal clearance or if other components of the adaptive immune system, such as IL-17-producing CD8+ T cells, are involved in antifungal host defense.

Therefore, in this chapter we examine the host response to *C. albicans* in the context of CD4+ T cell deficiency and explore the role of CD4-independent sources of IL-17 in antifungal immunity. It appears that CD8+ T cells induced upon re-challenge of CD4-/- mice play a previously unappreciated role in anti-*C. albicans* immunity.

4.2   RESULTS

4.2.1   CD4-/- mice are resistant to *C. albicans* re-challenge.

In order to determine if CD4+ T cells are an absolute requirement for antifungal host defense, CD4-/- mice were subjected to re-challenge. We monitored their weight over a period of 5 days post re-challenge, since weight loss is a good indicator of disease progression. We also plated tongue tissue for quantification of fungal burden on day 5 after 2° infection.   Unexpectedly, CD4-/- mice were resistant to re-challenge and completely cleared *C. albicans* from the tongue (**Figure 4.1**). This finding indicated that, in our OPC model, CD4+ T cells are not the sole requirement for *C. albicans* clearance. Furthermore, given the importance of IL-17 in antifungal host defense, these data also indicated that CD4+ T cells are not solely responsible for IL-17 production and further suggested the existence of compensatory sources of IL-17.

Figure 4.1: CD4-/- mice are resistant to OPC. WT and CD4-/- mice were re-challenged as described in **Figure 2.1** and their weight was monitored for 5 consecutive days following re-challenge. On day 5, the tongue was harvested and plated for colony enumeration (inset). Weight data is presented as percent of the original weight at the time of infection (Day 0).

4.2.2 CD8+ T cells from CD4-/-, but not WT, mice synthesize IL-17 after re-challenge in an antigen-specific manner.

The finding that CD4-/- mice were resistant to *C. albicans* infection prompted us to determine whether compensatory sources of IL-17 are present in CD4-/- mice during OPC. We found that in the absence of CD4+ T cells, CD8+ T cells synthesize IL-17 upon *C. albicans* re-challenge **(Figure 4.2)**. Importantly, as shown in **Figure 3.6 and 4.2,** CD8+ T cells from WT mice do not produce IL-17. We also detected CD8+IFNγ+ cells both in WT and CD4-/- mice but there was no difference between the mice that received a sham infection and the mice that were re-challenged.

In order to gain insight into the antigen specificity of these CD8+IL-17+ cells we stimulated total CD4-/- cLN cells with HK *C. albicans in vitro* following either 1° infection alone or 1° and 2° challenges. **Figure 4.3** shows that CD4-/- cLN cells produced as much IL-17 as WT cLN cells but only in the presence of HK *C. albicans*, demonstrating that IL-17 production depended on the presence of antigen. IFNγ was also present in these supernatant in negligible amounts **(Figure 4.3).** These data suggest that CD8+ T cells are a compensatory source of IL-17 in the context of CD4+ T cell deficiency.

In addition, we also tested the susceptibility of CD8-/- mice to *C. albicans* re-challenge. Since CD8-/- mice still possess CD4+ T cells presumably capable of producing IL-17 upon re-encounter with *C. albicans*, we expected CD8-/- mice to be resistant to re-challenge. Indeed, CD8-/- mice were completely resistant to 2° infection **(Figure 4.4)**. As expected, CD4+ T cells produced IL-17 upon secondary infection of both WT and CD8-/- mice **(Figure 4.5)**, which correlated with antigen-specific and CD4-dependent production of IL-17 upon re-stimulation of CD8-/- cLN cells with HK *C.*

albicans **(Figure 4.6)**. Small amounts of IFNγ were also observed in CD8-/- cLN cell supernatants.
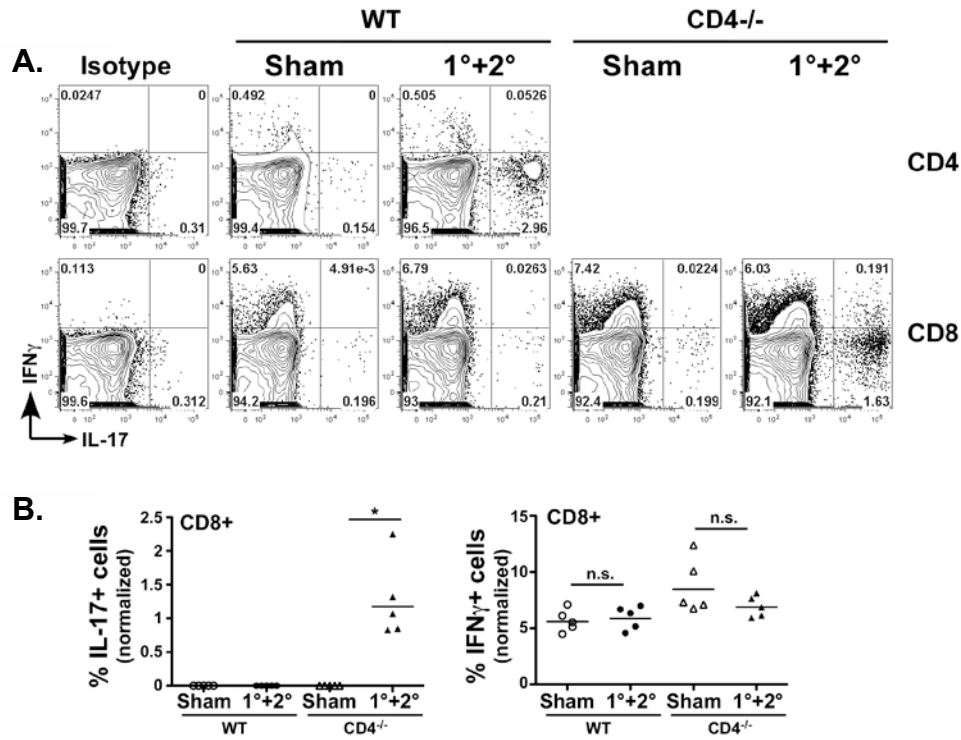
Figure 4.2: CD8+ T cells from CD4-/- mice produce IL-17 upon re-challenge. WT and CD4-/- mice were re-challenged as in **Figure 2.1** and the cLN were harvested after 5 days. Single cells suspensions of cLN cells were analyzed by flow cytometry for surface expression of CD3, CD4 and CD8 and intracellular IL-17 and IFNγ. Lymphocytes were gated on CD3+ cells and subsequently on CD4+ or CD8+. The frequencies of IL-17+ and IFNγ+ cells among CD4+ or CD8+ T cells is depicted in **(A)**. A summary the data corresponding to all the mice used in this experiment is shown in **(B)**.
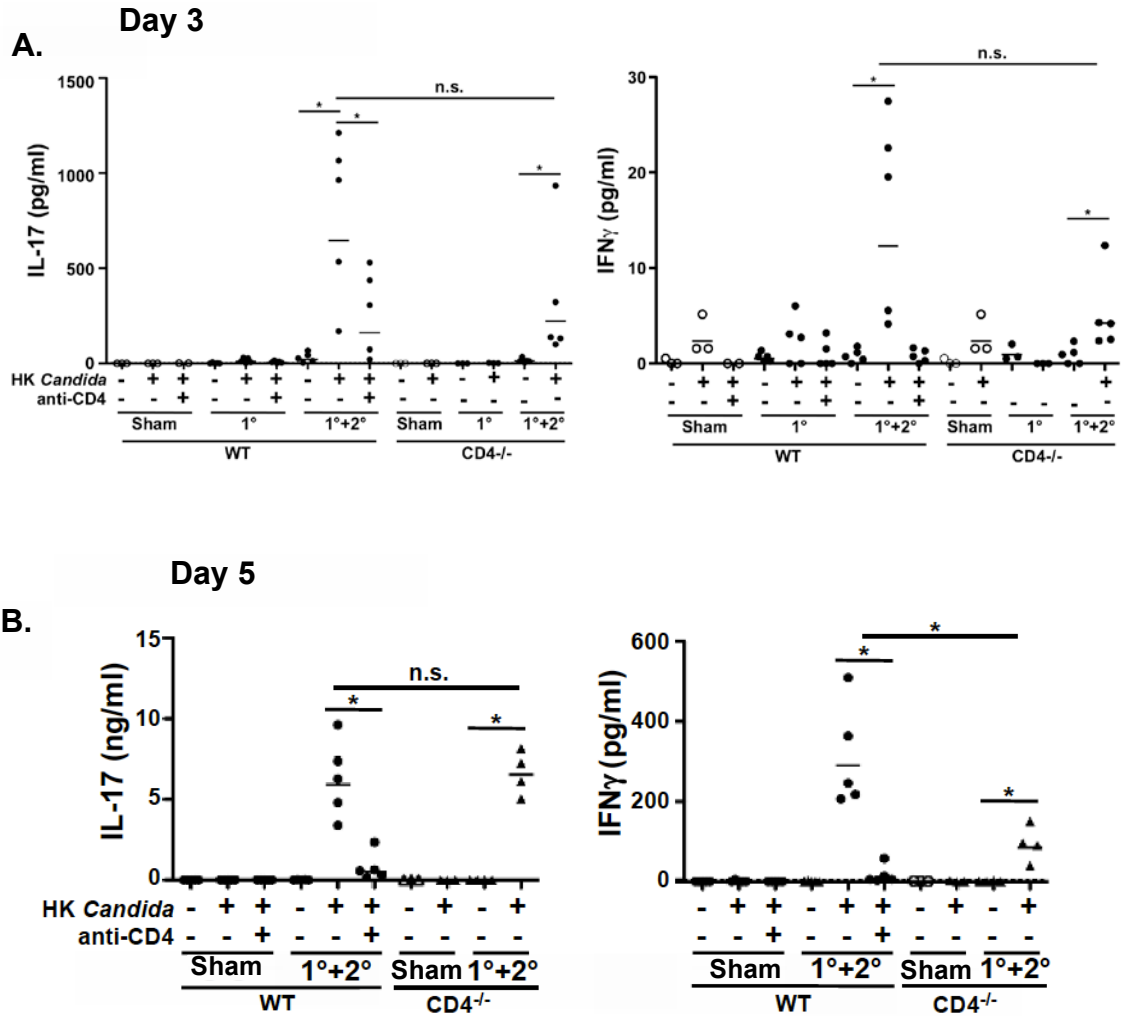
Figure 4.3: CD8+IL-17+ cells induced upon re-challenge of CD4-/- mice are antigen-specific. WT and CD4-/- mice were given a sham, 1° only or 1° + 2° infections and, after 3 **(A)** or 5 **(B)** days, cLN cells were isolated and cultured *in vitro* in the presence or absence of HK *C. albicans* +/- anti-CD4 blocking antibody (5µg/ml). Following a 5-day incubation period supernatants were harvested and analyzed for IL-17 and IFNγ production by ELISA. The data depicted in panels **(A)** and **(B)** resulted from two different experiments.
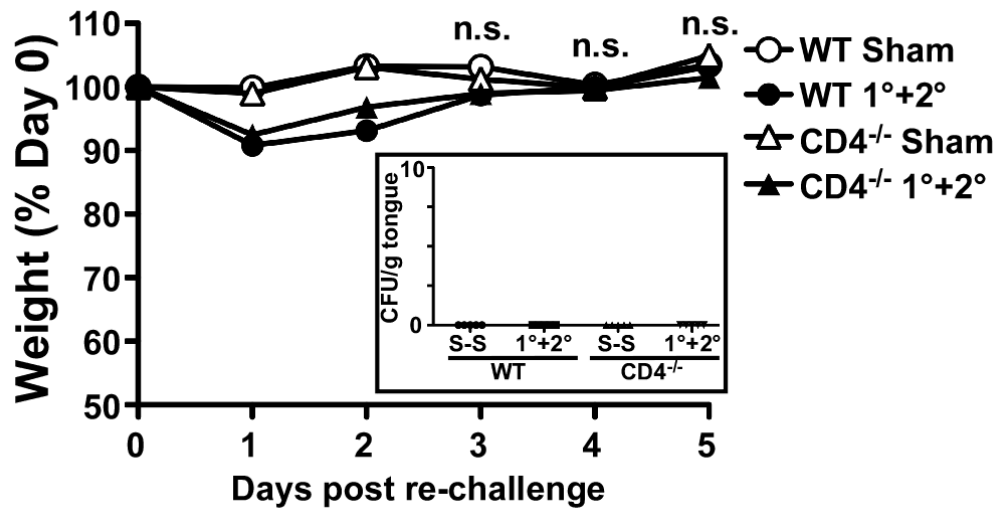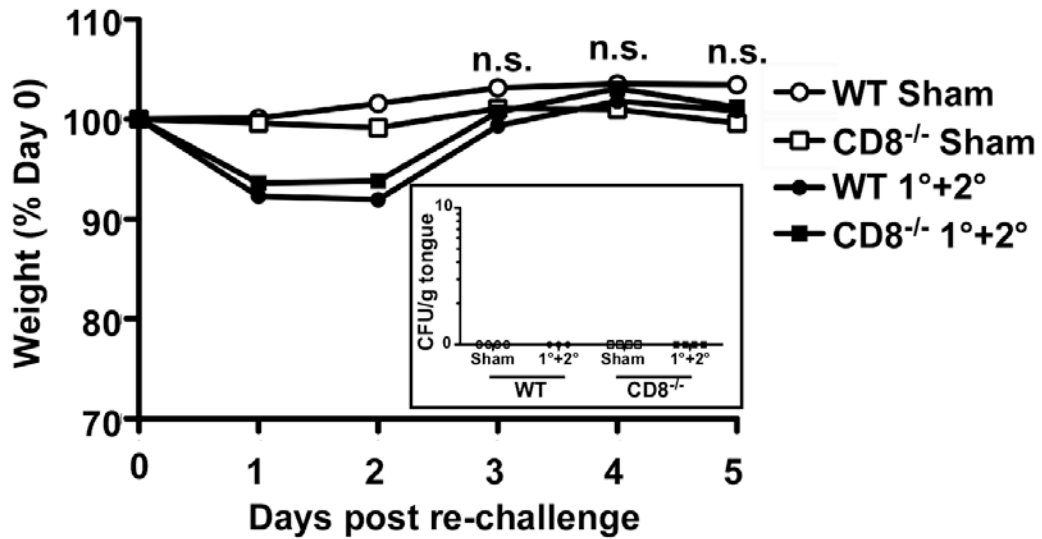
Figure 4.4: CD8-/- mice are resistance to OPC. WT and CD8-/- mice were re-challenged as described in **Figure 2.1** and their weight was monitored for 5 consecutive days following re-challenge. On day 5, the tongue was harvested and plated for colony enumeration (inset). Weight data is presented as percent of the original weight at the time of infection (Day 0).

A. Day 3
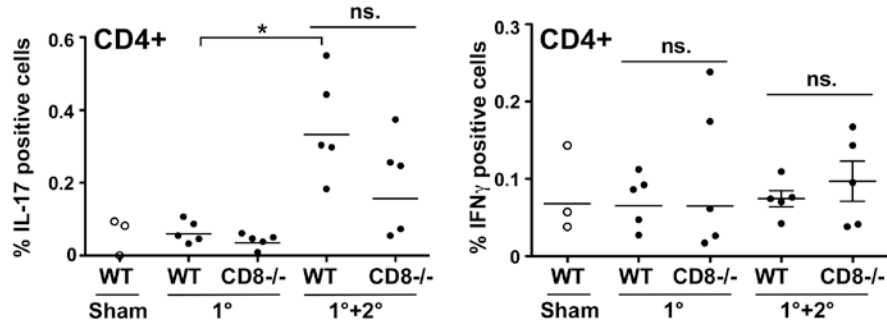
B. Day 5
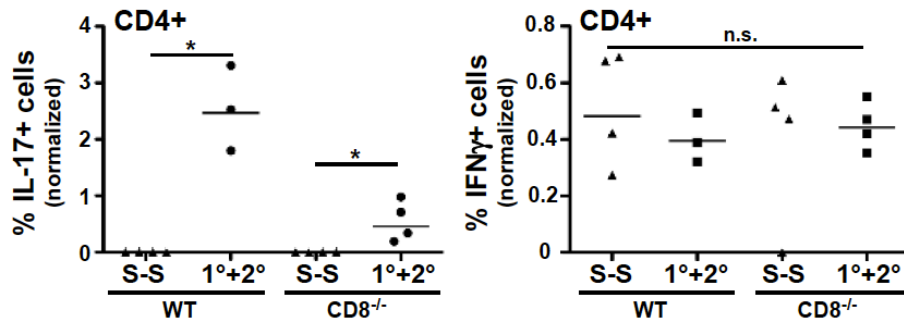
Figure 4.5: Frequency of CD4+Il-17+ cells in CD4-/- mice post re-challenge. WT and CD8-/- mice were re-challenged as in **Figure 2.1.** 3 to 5 days post-re-challenge cLN cells were analyzed for surface expression of CD4 and intracellular IL-17 and IFNγ expression. Cells were gated on lymphocytes, CD3 and CD4. The frequency of IL-17+ or IFNγ+ cells among CD4+ T cells is depicted. Data was normalized to isotype controls. The data depicted in panels **(A)** and **(B)** resulted from two different experiments.
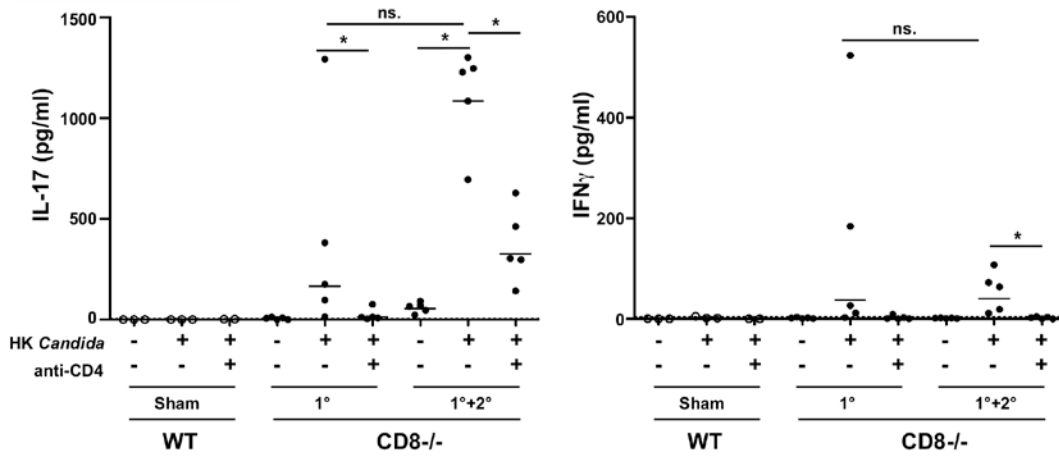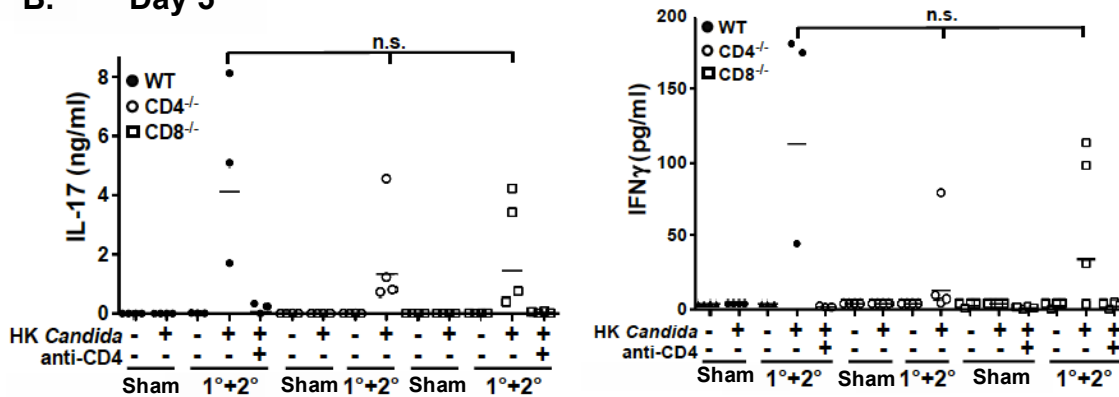
Figure 4.6: CD4+ T cells from CD8-/- mice produce IL-17 in an antigen-specific and CD4-dependent manner. WT, CD4-/- and CD8-/- mice were given a sham, 1° only or 1° + 2° infections and, after 3 **(A)** or 5 **(B)** days, cLN cells were isolated and cultured *in vitro* in the presence or absence of HK *C. albicans* +/- anti-CD4 blocking antibody (5µg/ml). Following a 5-day incubation period supernatants were harvested and analyzed for IL-17 and IFNγ production by ELISA. The data depicted in panels **(A)** and **(B)** resulted from two different experiments.

4.2.3 Antibody-mediated depletion of CD4+ and CD8+ T cells in WT mice does not result in susceptibility to *C. albicans* re-challenge.

The use of CD4-/- and CD8-/- mice allowed us to address whether or not compensatory sources of IL-17 were present during re-challenge. However, we did not answer the question of whether or not simultaneous depletion of both CD4+ and CD8+ T cells confers susceptibility to OPC. An adequate way to address this question was to re-challenge CD4 and CD8 double knockout (CD4CD8-/-) mice and quantify fungal burden in tongue tissue following re-challenge but these mice were not available at the time these experiments were performed. We then decided to deplete CD4+ and CD8+ T cells *in vivo* via injection of depleting antibodies prior to re-challenge. WT, CD4-/- and CD8-/- mice depleted of CD4+ or CD8+ T cells alone or simultaneously were resistant to *C. albicans* re-challenge **(Figure 4.7)**. This was not due to defective cell depletion, as surface staining of splenocytes 5 days after the antibodies were injected demonstrated complete depletion of CD4+ and CD8+ T cells **(Figure 4.8)**. However, it is certainly possible that the antibody did not reach the oral cavity and thus, local cell depletion was not as effective as peripheral cell depletion, but this hypothesis was not tested.

Our findings confirmed the results presented in **Figures 4.1** and **4.4** and indicate that even in the context of combined CD4+ and CD8+ T cell depletion mice are still resistant to OPC. This suggested that, in addition to CD8+ T cells, other compensatory sources of IL-17 exist and "take over" IL-17 production when CD4+ T cells are absent. Furthermore, this also points to the importance of innate sources of IL-17, which are

presumably operating in the absence of CD4+ and CD8+ T cells, in host defense against OPC.
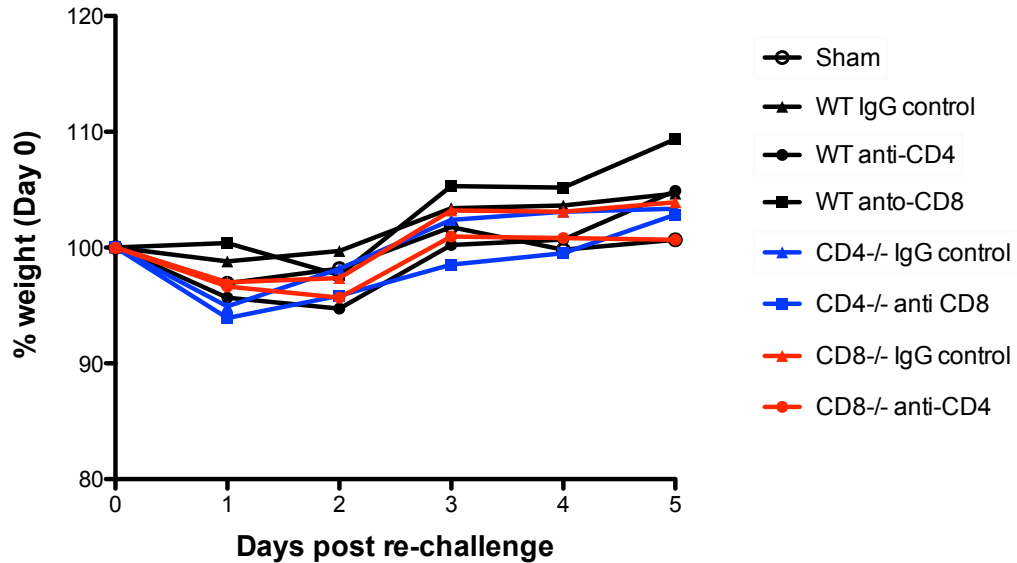
Figure 4.7: Simultaneous depletion of CD4+ and CD8+ T cells results in resistance to OPC. WT, CD4-/- and CD8-/- mice were re-challenged and depleted of CD4+ and/or CD8+ T cells as described in **Figures 2.1 and 2.4**, respectively. Weight was monitored everyday for a period of 5 days after re-challenge. Data is presented as percent of initial weight at the moment of secondary challenge (Day 0).

Injected antibody

| Isotype | Anti-CD4 | Anti-CD8 |
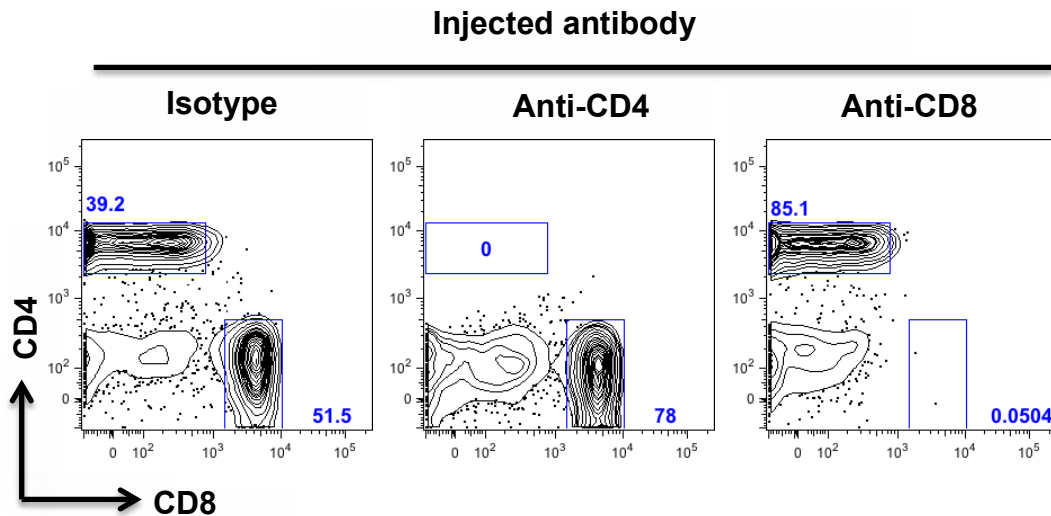
Figure 4.8: In vivo CD4+ and CD8+ T cell depletion is effective. CD4+ and CD8+ T cells were depleted as described in **Figure 2.4** and splenocytes were harvested 5 days later and analyzed by flow cytometry for surface expression of CD4 and CD8. The frequency of CD4+ and CD8+ cells among lymphocytes is depicted.

4.2.4 CD8+ T and CD4-CD8- (DN) cells from CD4-/-, but not WT, mice re-challenged with *C. albicans* protect Rag1-/- mice from OPC.

In light of the apparent resistance of CD4-/-, CD8-/- and T cell-depleted mice to OPC and considering the possibility that potent innate IL-17 responses were masking the role of adaptive IL-17 responses in OPC, it was important to determine whether *C. albicans*-primed CD8+IL-17+ cells protected susceptible hosts from OPC. Therefore, we isolated CD4+, CD8+ and DN cells from WT and CD4-/- mice 3 days after re-challenge and transferred them into Rag1-/- mice 1 day prior to infection (outlined in **Figure 2.5**). We

then enumerated *C. albicans* colonies in the tongue of Rag1-/- recipients 4 days following infection. Since cLN cells from CD4-/- mice produce IL-17 at levels equivalent to CD4+ cLN cells from WT mice, we hypothesized that the transfer of CD8+ T cells from CD4-/- mice following re-challenge would fully protect of Rag1-/- mice from OPC. As expected, CD4+ T cells from WT mice conferred Rag1-/- mice with complete protection from OPC **(Figure 4.9)**. CD8+ T cells from CD4-/- mice offered some protection but not to the same extent that CD4+ T cells from WT mice. Surprisingly, the DN cell fraction from CD4-/-, but not WT, mice completely protected Rag1-/- mice from OPC, suggesting that in addition to CD8+ T cells, other sources of IL-17 can compensate for the absence of CD4+ T cells.

4.2.5 Protective CD4+, CD8+ and DN cells home to the tongue after transfer into Rag1-/- mice.

The protection conferred to Rag1-/- mice by CD4+ T and CD8+ T or DN cells isolated from WT and CD4-/- mice, respectively, after re-challenge demonstrated that *Candida*-primed cells home to the tongue during infection and actively clear the fungus **(Figure 4.9)**. In order to gain more insight into the local immune response during infection, we measured the mRNA levels of CD3, CD4 and CD8 in the tongue of Rag1-/- recipients 4 days following infection post-transfer. Interestingly, CD3, CD4 and CD8 mRNA levels were increased when the transferred population was associated with protection from OPC **(Figure 4.10)**, suggesting that these cells home to the tongue after transfer and perhaps expand *in situ* when they re-encounter *C. albicans.*

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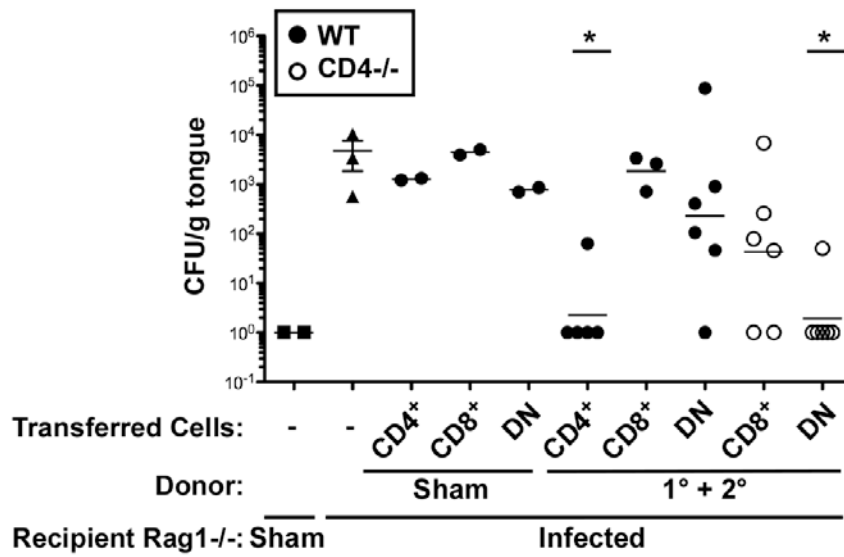
Figure 4.9: CD8+ T and DN cells from CD4-/- mice protect Rag1-/- mice from OPC. Total CD4+, CD8+ and DN cells were isolated from WT and CD4-/- mice and transferred into Rag1-/- mice as described in **Figure 2.5** 1 day prior to infection. 4 days after infection *C. albicans* colonies were enumerated in the tongue of recipient Rag1-/- mice.
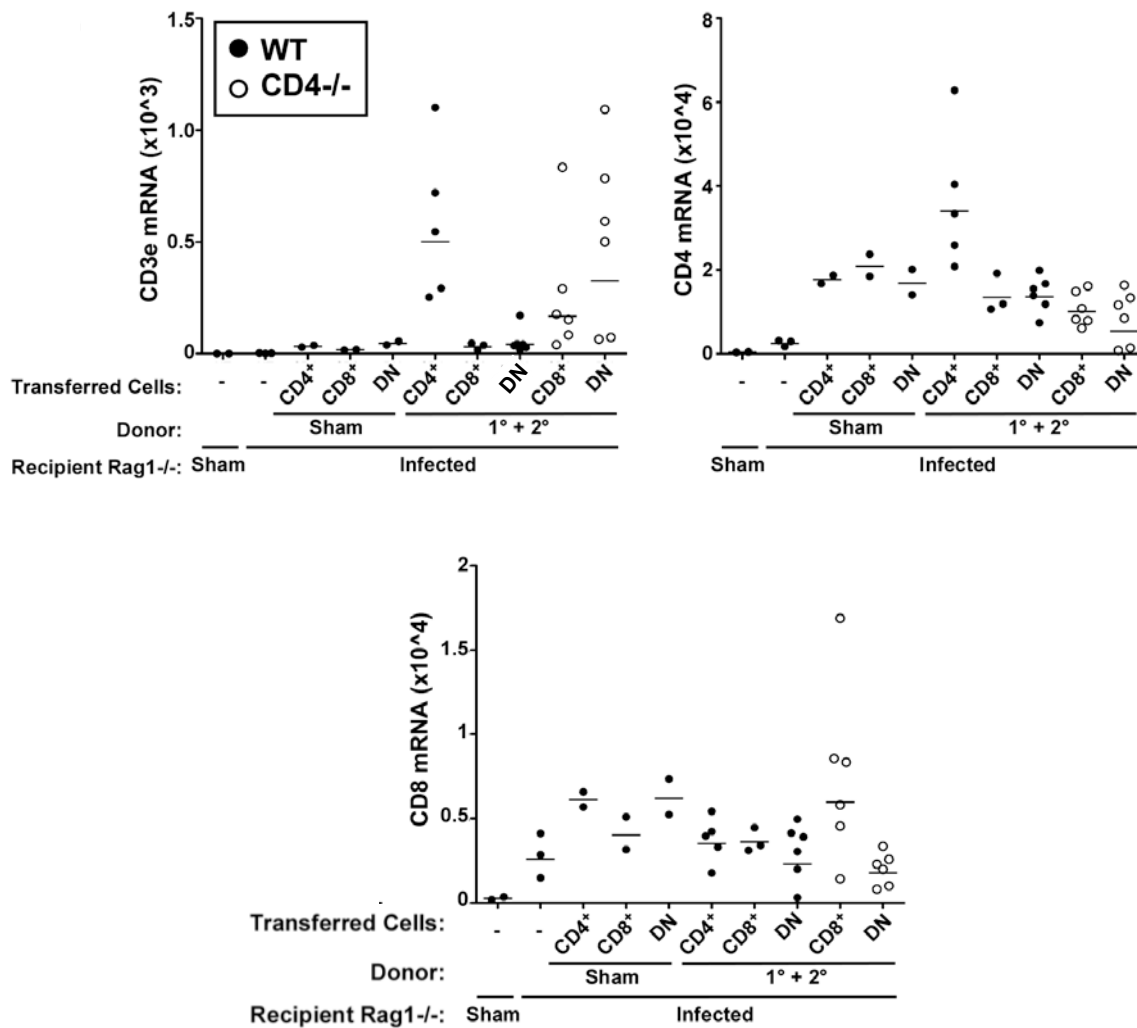
Figure 4.10: Protective CD4+, CD8+ and DN cells home to the tongue upon transfer into Rag1-/- mice. Total CD4+, CD8+ and DN cells were isolated from WT and CD4-/- mice and transferred into Rag1-/- mice as described in **Figure 2.5** 1 day prior to infection. 4 days after infection the tongue RNA was isolated and CD3e, CD4 and CD8 mRNA levels were quantified by real-time PCR.

4.2.6 Phenotype of protective DN cells.

The protective effect of DN cells isolated from CD4-/- mice after re-challenge prompted us to study their phenotype in further detail. The experiment depicted in **Figure 4.10** showed that DN cells are CD3+. This finding was subsequently confirmed in an independent experiment where CD3+DN cells produced IL-17 upon re-challenge only in CD4-/- mice **(Figure 4.11A)**. Of note, we detected no infection-dependent IL-17 production in WT or CD4-/- CD3- cells **(Figure 4.11B)**. The only other CD3+ population in the lymph nodes is γδ T cells (Turchinovich and Pennington, 2011). In addition, a population of MHC class II-restricted CD4- T cells has been described in CD4-/- mice (Tyznik et al., 2004). Thus, we examined surface expression of γδ and αβ on CD3+DN cells from WT and CD4-/- mice 3 days following re-challenge. We also included CD8-/- mice in this experiment to determine whether a similar DN cell population was present in the context of CD8+ T cell deficiency. We found no difference in the frequency of γδ and αβ CD3+DN cells among total cLN cells from WT, CD4-/- and CD8-/- mice following re-challenge **(Figure 4.11C-D)**. In addition, we measured γδ and αβ mRNA levels in the tongue of Rag1-/- mice that received DN cells from re-challenged CD4-/- mice and were subsequently infected with *C. albicans.* We found no obvious trend in γδ or αβ expression that will direct us to the phenotype of DN cells **(Figure 4.12)**. Therefore, it appears that the only apparent phenotypic characteristic of these cells is their expression of CD3.

Figure 4.11

Figure 4.11 cont.

C.

D.
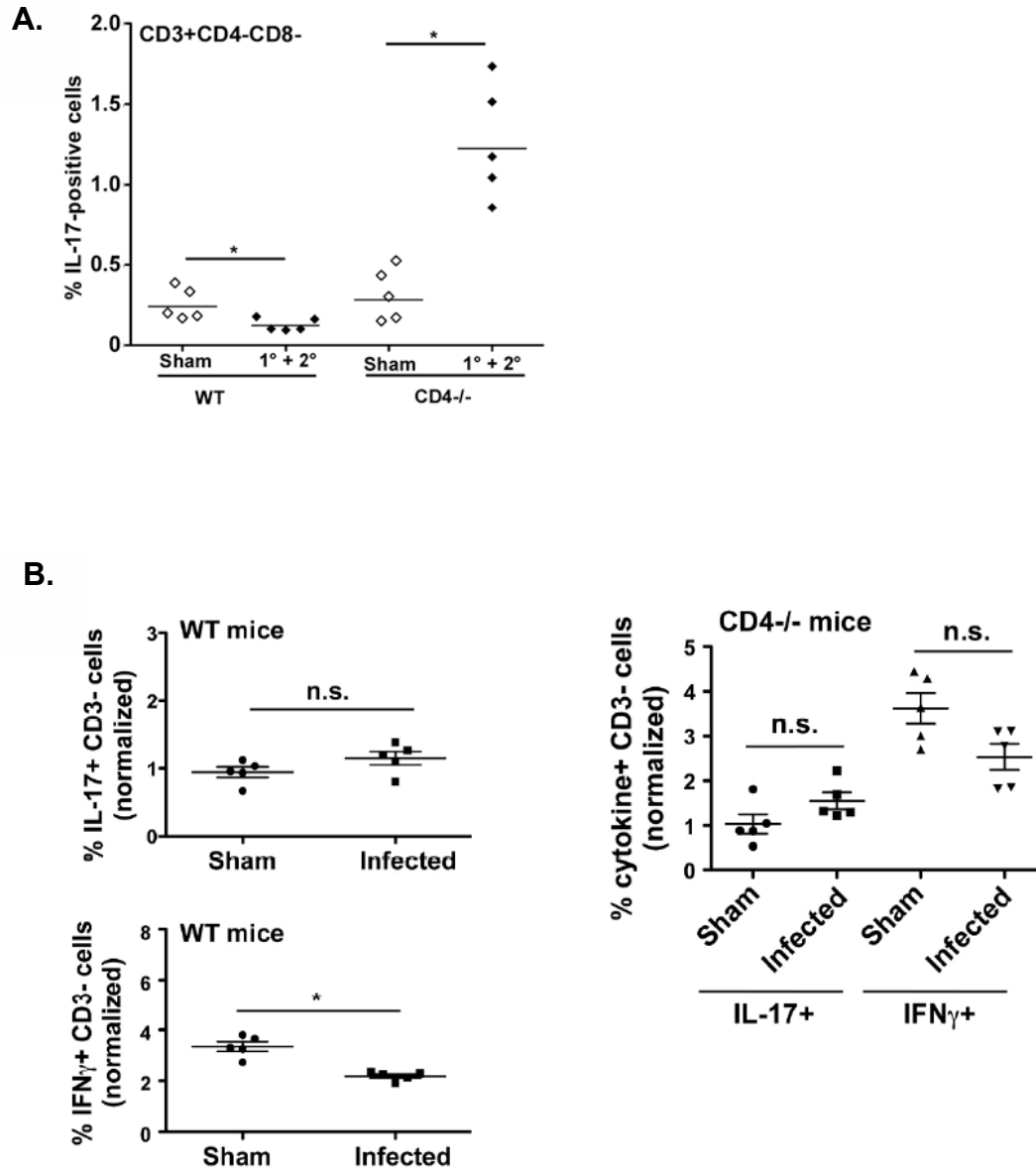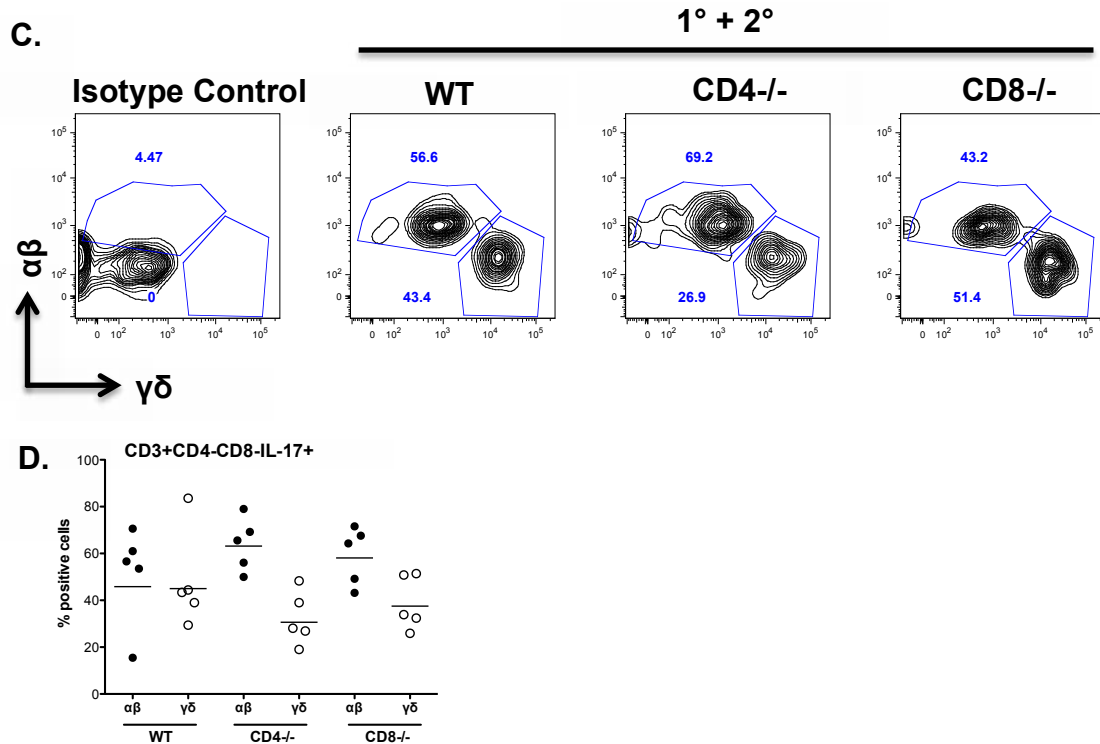
Figure 4.11: CD3, αβ TCR and γδ TCR expression on DN cells. WT and CD4-/- mice were re-challenged and after 5 days the cLN were analyzed for surface expression of CD3, CD4 and CD8 and intracellular IL-17 and IFNγ. Cells were gated on lymphocytes, CD4-CD8- and CD3+ cells. The frequency of IL-17+ cells among CD3+CD4-CD8- is depicted **(A).** Alternatively cells were gated on lymphocytes and CD3- cells and the frequencies of IL-17+ and IFNγ+ cells are depicted **(B).** WT, CD4-/- and CD8-/- mice were re-challenged and after 3 days cLN were analyzed for expression of αβ and γδ TCR among CD3+IL-17+ cells. Representative plots are depicted in **(C)** and the summary of the cumulative data is presented in **(D).** Experiments in panels **(A)**, **(B)** and **(C-D)** resulted from 3 separate experiments.
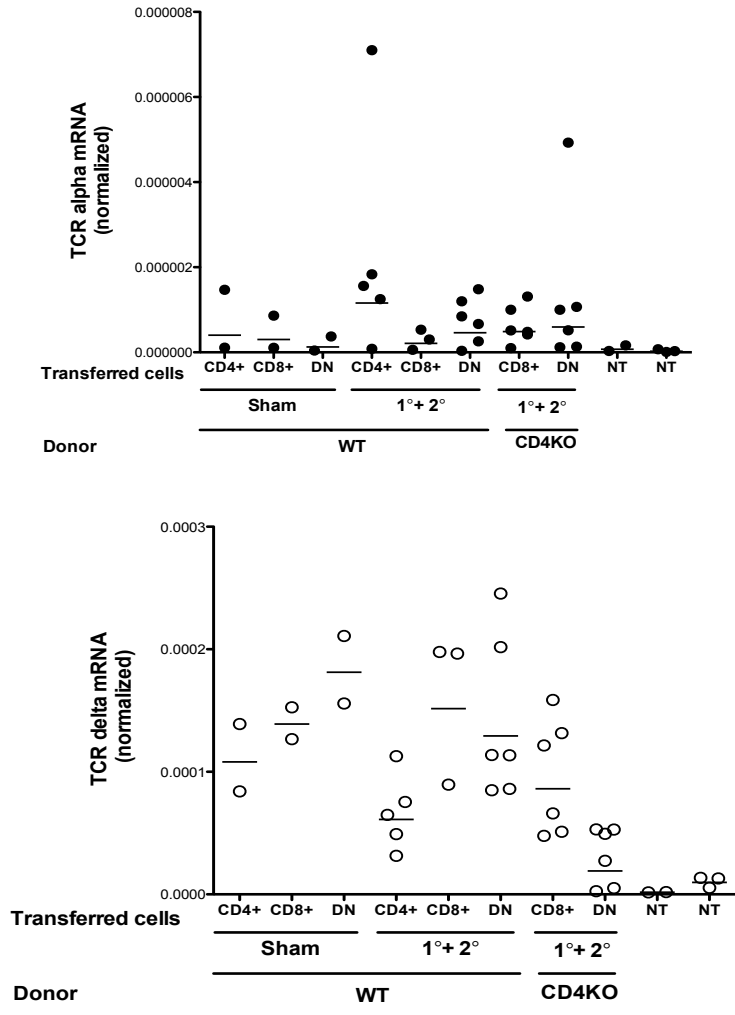
Figure 4.12: αβ and γδ TCR expression in the tongue of infected Rag1-/-mice after adoptive transfer of *Candida*-primed CD4+ T, CD8+ T and DN cells. Rag1-/- mice were adoptively transferred as described in Figure 2.5 with either naïve or Candida-primed CD4+ T, CD8+ T or DN cells 1 day prior to infection. After 4 days the tongue of recipient Rag1-/- mice was analyzed for expression of αβ and γδ TCR.

4.3 DISCUSSION

In this chapter we showed that CD4-/- mice were resistant to OPC and that this was associated with the presence of antigen-specific CD8+IL-17+ cells, demonstrating a novel compensatory mechanism that comes into action when Th17 cells are absent. Furthermore, *Candida*-primed CD8+ T cells isolated from CD4-/- mice protected susceptible Rag1-/- mice from OPC. In addition, we found that CD3+DN cells from CD4-/- and not WT mice also protected Rag1-/- hosts. These data demonstrated that, in the absence of Th17 cells, other cell types can "take-over" IL-17 production and contribute to fight infection. However, the presence of CD8+IL-17+ T cells alone did not account for the resistance of CD4-/- mice to *C. albicans* infection because depletion of CD8+ T cells in these mice did not render them susceptible to OPC. We attributed this phenomenon to the presence of strong innate IL-17 sources that functioned to clear *C. albicans* infection. Whether or not the protective CD3+DN cell population "fits" the profile of an innate cell is uncertain and is an important area of future inquiring.

Our attempts to identify the phenotype of protective DN cells isolated from CD4-/- mice were inconclusive. We established they were CD3+ but did not determine if they were αβ+ or γδ+. Flow cytometry data indicated that approximately 40-60% and 30-40% of CD3+IL-17+DN cells were αβ+ and γδ+, respectively. However, there were no differences in the frequency of αβ+ or γδ+ CD3+IL-17+DN cells between re-challenged WT and CD4-/- or CD8-/- deficient mice. Thus, in contrast to CD3 expression on IL-17+DN cells, αβ and γδ TCR expression was not an exclusive trait of DN cells from CD4-/- mice. Perhaps a better approach to defining the phenotype of this cell type is to

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stain the tongue for γδ and αβ TCRs via immunofluorescence after transfer of DN cells from CD4-/- mice post re-challenge into Rag1-/- hosts. In spite of the technical challenges of defining the phenotype of this cells, we know they home to the tongue and protect Rag1-/- mice from OPC, which stresses their importance as compensatory sources of IL-17 in CD4-/- mice.

It was surprising to find compensatory production of IL-17 by CD8+ T cells in CD4$^{-/-}$ mice and, even more importantly, that these CD8+IL-17+ cells protected Rag1-/- mice from OPC. This finding is supported by recent studies in a vaccine model of *Blastomyces dermatitidis* infection that described the presence of antigen-specific Tc17 cells induced upon vaccination in the absence of CD4+ T cell help (Nanjappa et al., 2012a; Nanjappa et al., 2012b). Remarkably, vaccination-induced Tc17 cells protected mice from lethal pulmonary challenge. This has important implications in the development of antifungal vaccines to benefit immunocompromised individuals, such as HIV+ patients, where residual immunity must be harnessed to fight opportunistic infections. Interestingly, studies in HIV+ individuals with OPC revealed that CD8+ T cells migrated to the lamina propria of the tongue but, due to a defect in E-cadherin expression, did not reach the outer epithelium where *C. albicans* resided (McNulty et al., 2005; Myers et al., 2003). These studies suggested that CD4+ T cells constitute the primary line of defense against *C. albicans* but when counts fall below the protective threshold (200 cells/mm^3) CD8+ T cells, along with epithelial cells, can then act as the "reserve warriors" to fight *C. albicans* (Fidel, 2011). These CD8+ T cells that migrated to the tongue were activated memory cells but their cytokine profile was not defined. Studies in non-human primates indicated that Tc17 cells were present at early stages of

SIV infection but their numbers reduced dramatically during end-stage AIDS (Nigam et al., 2011). However, no information was provided regarding the OPC status of these SIV+ monkeys and its correlation to the frequency of Tc17 cells. Therefore, Tc17 represent a previously underappreciated component of antifungal immunity that should be targeted to design vaccines that would benefit both healthy and immune compromised individuals.

Taken together, these findings demonstrate that although antigen-specific Th17 responses contribute to fungal clearance, their absence is compensated for by CD8+IL-17+ and CD3+DN cells in CD4-/- mice. Moreover, these compensatory sources of IL-17 protected susceptible Rag1-/- mice from OPC and thus, constitute important components of antifungal immunity under immune suppressive conditions.

5.0   CHAPTER FIVE: MECHANISMS OF IL-17-MEDIATED IMMUNITY

5.1 BACKGROUND

The mechanisms of IL-17 mediated immunity are associated to the induction of antimicrobial peptides (AMP) in epithelial surfaces and the mobilization of neutrophils to peripheral inflammatory sites. Pioneering studies of IL-17 in a *Klebsiella pneumoniae* infection model demonstrated that IL-17 is a major inducer of granulopoiesis and neutrophil chemotaxis (Ye et al., 2001). A plethora of studies in other infection systems, many conducted long before the recognition of Th17 cells, showed that IL-17 mobilizes neutrophils. IL-17 activates neutrophils indirectly, primarily by upregulating expression of G-CSF and CXC chemokines in mucosal epithelial cells as well as the local stroma (Khader et al., 2009). With respect to candidiasis, IL-17 appears to mediate many of its protective effects via mobilization of neutrophils to peripheral inflammatory sites. In systemic candidemia, IL-17R-deficient mice exhibited decreased absolute neutrophil counts in peripheral blood, which correlated with impaired neutrophil recruitment and myeloperoxidase activity in the kidney (Huang et al., 2004). In OPC, both IL-23p19-/- and IL-17R-/- mice exhibit decreased neutrophil numbers in the oral mucosa compared to resistant mouse strains. Moreover, microarray analysis of tongue tissue showed induction of prototypical IL-17 target genes that serve to expand or recruit neutrophils, such as genes encoding CXCL1 (KC, Groα), CXCL2 (MIP2), CXCL5 and CSF3 (G-CSF) (Conti et al., 2009). In addition, *C. albicans* can stimulate oral epithelial cells to secrete CXCL8 (IL-8), another potent neutrophil chemoattractant (Dongari-Bagtzoglou and Kashleva, 2003; Dongari-Bagtzoglou et al., 2005). Similarly, in a murine model of VVC 75% of the total vaginal infiltrate at 48 hours post-infection were found to be neutrophils, coinciding with elevated IL-17 and IL-23 in vaginal fluid (Pietrella et al.,

2011). In addition, it has been demonstrated that β-glucans can induce human neutrophil chemotaxis and that neutrophil extracellular traps can capture and kill both *C. albicans* yeast and hyphae (Urban et al., 2006). Therefore, IL-17-dependent recruitment of neutrophils to the site of infection is likely an important element in host responses to *C. albicans.*

Despite its potent effects on neutrophils, is not clear whether mobilization of neutrophils is the primary underlying mechanism by which IL-17 mediates antifungal effects. Although patients undergoing chemotherapy associated with neutropenia are highly susceptible to various forms of disseminated candidiasis, individuals with isolated neutropenia or neutrophil defects such as chronic granulomatous disease (CGD) are not particularly prone to *Candida* infections (Del Favero, 2000; Grigull et al., 2006; Maertens et al., 2001). In this regard, IL-17 induces antimicrobial peptide (AMP) expression in a variety of settings, and many of the IL-17-induced AMPs have direct antifungal activity towards *Candida* (Gorr, 2009). In skin, IL-17 signals cooperatively with IL-22 to induce AMPs such as S100A7 and β-defensin 2 (BD2) in keratinocytes and epithelial cells (Liang et al., 2006). In humans with HIES, this effect is significantly impaired (Minegishi et al., 2007), perhaps explaining the susceptibility to dermal and oral candidiasis in these patients. In VVC, production of BD2 by vaginal epithelial cells is inhibited following ablation of Th17 responses (Pietrella et al., 2011). Moreover**,** in murine OPC, AMP such as S100A8, S100A9 and β-defensin 3 (murine homologue of BD2) are induced in tongue in WT mice but impaired in IL-17R-/- animals (Conti et al., 2009). Thus it appears that IL-17 mediates its effects primarily by inducing recruitment of neutrophils to sites of infection and stimulating production of antimicrobial peptides

but which is more important remains unclear to date.

5.2   RESULTS

5.2.1 Expression of IL-17 target genes and neutrophil infiltrate in the tongue after re-challenge.

In light of the important role of IL-17 in the induction of antimicrobial peptides and in neutrophil homeostasis and chemotaxis, we compared the expression of various genes associated with these processes during primary infection and re-challenge. To this end, mice were subjected to 1° infection alone or followed by secondary challenge and the tongue tissue was subsequently harvested at days 1,2 and 3 post re-challenge. As a negative control, we included a cohort of naïve mice that received a mock infection. Tongue RNA was then extracted and used for cDNA synthesis, which was followed by real-time PCR. We observed that Csf2 (GM-CSF), Csf3 (G-CSF), CXCL1 and CXCL5, which are involved in granulopoiesis and neutrophil chemotaxis, were induced on day 1, started declining on day 2 and were almost undetectable by day 3 **(Figure 5.1)**. On the other hand, antimicrobial peptides, such as β-defensin 3 and S100A9, were induced on days 2 and 3 after both primary infection and re-challenge **(Figure 5.1)**. No statistical differences in gene expression were observed between the mice that received primary infection alone and those that were subjected to re-challenge. Accordingly, no obvious differences were observed in the recruitment of neutrophils to the tongue following 1° infection only or 1° + 2° challenge as observed by H & E staining of tissue sections

(Figure 5.2). Therefore, although Th17 responses accelerate *C. albicans* clearance **(Figure 3.3)** and protect susceptible host from OPC, adaptive IL-17 responses are superimposed by potent antifungal innate responses.
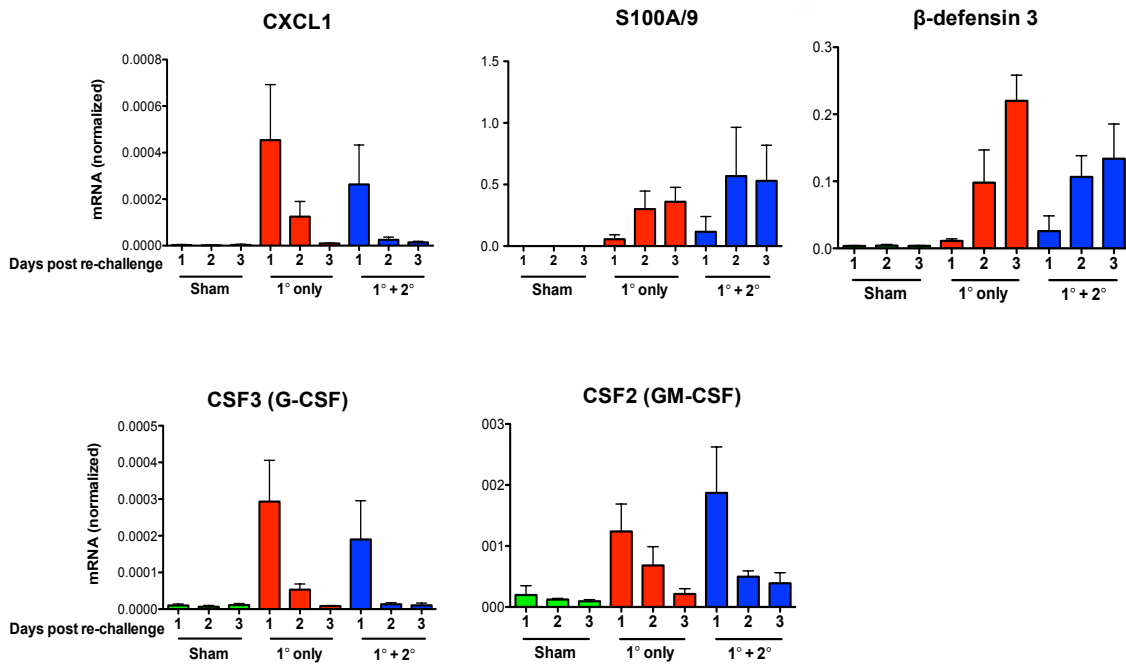
Figure 5.1: Expression of IL-17 target genes in tongue tissue during *C. albicans* re-challenge. Mice were given a sham, 1° only or 1° + 2° infections and the tongue was subsequently harvested at days 1, 2 and 3 post re-challenge. Tongue RNA was then isolated and analyzed for mRNA levels of the indicated genes. Data was normalized to GAPDH mRNA levels.

Figure 5.2

Day 1

Day 2

Figure 5.2 cont

Day 3
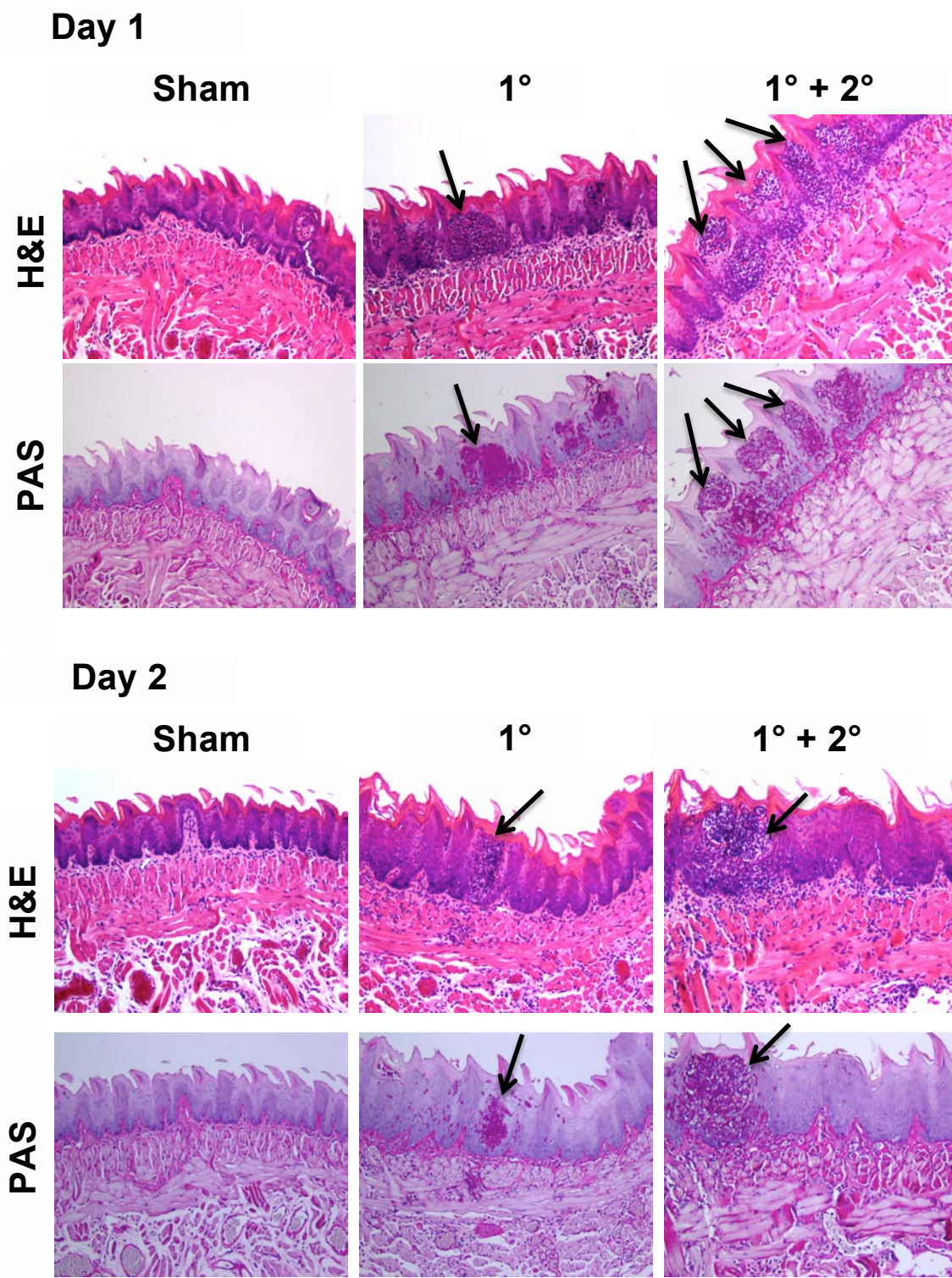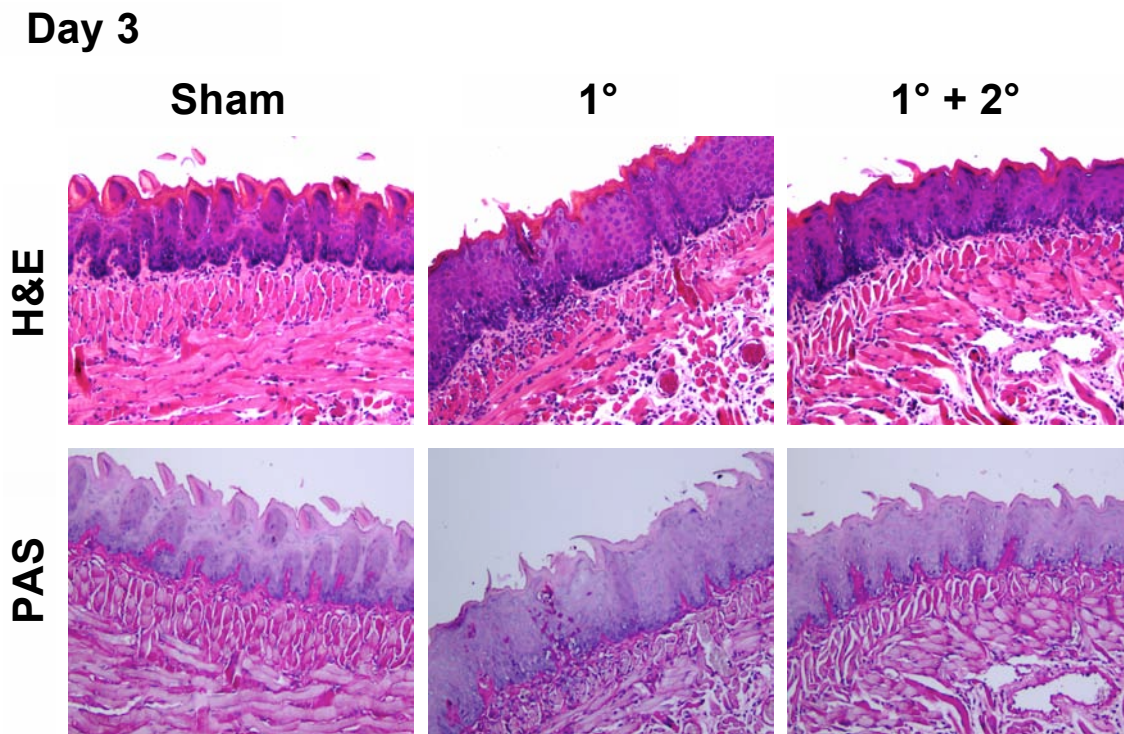
Figure 5.2: Histology sections of tongue tissue during *C. albicans* re-challenge. Mice were given a sham, 1° only or 1°+ 2° infections and, at days 1-3 post re-challenge, the tongues were harvested, preserved in NBF and parafilm embedded for subsequent tissue sectioning. Tissue sections were then stained with H&E for visualization of tongue-infiltrating neutrophils and PAS for visualization of *C. albicans* yeast and hyphae. Tongue lesions are indicated with black arrows.

5.3   DISCUSSION

In chapter 1, we determined that pre-exposure to *C. albicans* resulted in accelerated fungal clearance, which was associated with an increase in the frequency of CD4+IL-17+ cells. Here we sought to determine whether differential induction of IL-17 target genes during re-challenge, in comparison to primary infection alone, could account for the 1 log decrease in fungal burden observed at days 1 and 2 post 2° infection. We found no statistical differences in gene expression between the mice that underwent 1° infection only and those that received 1° and 2° challenges. This correlated with a similar neutrophil infiltrate in both cohorts at all the time points examined following infection.

The fact that we did not detect any significant differences in gene expression upon re-challenge may be due to the potent innate IL-17 responses that superimpose on Th17-mediated immunity. Therefore, anti-*Candida* innate responses may be "masking" the contribution of *C. albicans*-specific CD4+ T cell responses.

Although there were no apparent differences in gene expression upon either primary infection alone or re-challenge, the gene expression patterns suggests that the first "wave" of genes targets neutrophil recruitment to the tongue, whereas the second "wave" results in secretion of antimicrobial peptides. However, to define these patterns in detail, a more extensive range of IL-17 target genes involved in neutrophil chemotaxis and antimicrobial peptide production needs to be examined.

6.0   CHAPTER SIX: CONCLUSIONS AND FUTURE DIRECTIONS

6.1   SUMMARY AND CURRENT MODEL OF IMMUNITY TO *C. ALBICANS* IN OPC

Our studies demonstrated that pre-exposure to *C. albicans* accelerated fungal clearance following re-challenge by causing a ~1 log decrease in fungal burden. This was associated with an increase in the frequency of antigen-specific CD4+IL-17+ cells, suggesting the involvement of classic Th17 cells in fungal eradication from the tongue. Surprisingly, however, CD4-/- mice were resistant to OPC, which appeared to be associated, at least in part, with the generation of CD8+IL-17+ and CD3+DN IL-17+ cells. Disease resistance in CD4-/- mice could not be exclusively attributed to the presence of CD8+IL-17+ because mice depleted of both CD4+ and CD8+ cells are also resistant to OPC. Thus, potent innate IL-17 responses appeared to be responsible for resistance to disease. It is known that this IL-17-producing innate cell requires a rearranged receptor because Rag1-/- mice are susceptible to OPC. In the absence of this innate component, such as in Rag1-/- mice, CD4+ and CD8+ T cells from WT and CD4-/- mice, respectively confer protection from OPC. This suggests that, in spite of the evident reduction in fungal burden following pre-exposure to *C. albicans,* innate IL-17-mediated immune responses are masking the role of classic antigen-specific Th17 cells. This was reflected in both neutrophil recruitment and gene expression profiles following either 1° infection alone or re-challenge. Neutrophils were recruited to the site of infection on days 1 and 2 after 1° infection or 1° + 2° challenges. In addition, in both cases (1° only or 1° + 2°) IL-17 target genes were expressed in two "waves". The first "wave" consisted CXCL1, CSF2 and CSF3 with the highest level of expression on day 1, starting to decline on day 2 and almost completely absent on day 3. The opposite was true for the second "wave" of genes, which consisted of antimicrobial peptides such

as S100A/9 and β-defensin 3; they were expressed at the lowest level on day 1 and peaked at day 3. Therefore, regardless of the source of IL-17, whether innate alone or accompanied by adaptive Th17 responses, the mechanisms of IL-17-mediated immunity appear to be the same.

In light of these findings, we believe that innate immune responses alone are capable of clearing *C. albicans* from the oral cavity. Upon pre-exposure to *C. albicans* and thus, the development of adaptive Th17 responses, innate and adaptive responses "team-up" to eradicate *C. albicans,* resulting in accelerated fungal clearance **(Figure 6.1).**

These data highlights the importance of animal models that better resemble the human condition, where *C. albicans* is a commensal and, as a result, the immune system is chronically exposed to the fungus. This constant access to antigen likely plays an important role in shaping anti-*Candida* T cell responses, which are largely Th17 mediated. Another important aspect regarding the commensal nature of *C. albicans,* is the other constituents of the human microflora. It is known that DCs in the lamina propria of the gut extend projections through the epithelium, which allows them to potentially interact with commensal microorganisms. Although the mechanisms by which the rich intestinal immune system maintains tolerance to commensal microbes while simultaneously providing immunity to infections remains unclear, it is possible that these DC-commensals interactions play a role in shaping host immune responses to pathogens (Iwasaki and Medzhitov, 2004). The fact that *C. albicans* is both a commensal and a pathogen (under conditions of immunosuppression) adds an

additional and intriguing level of complexity to the study of *C. albicans*-specific responses.
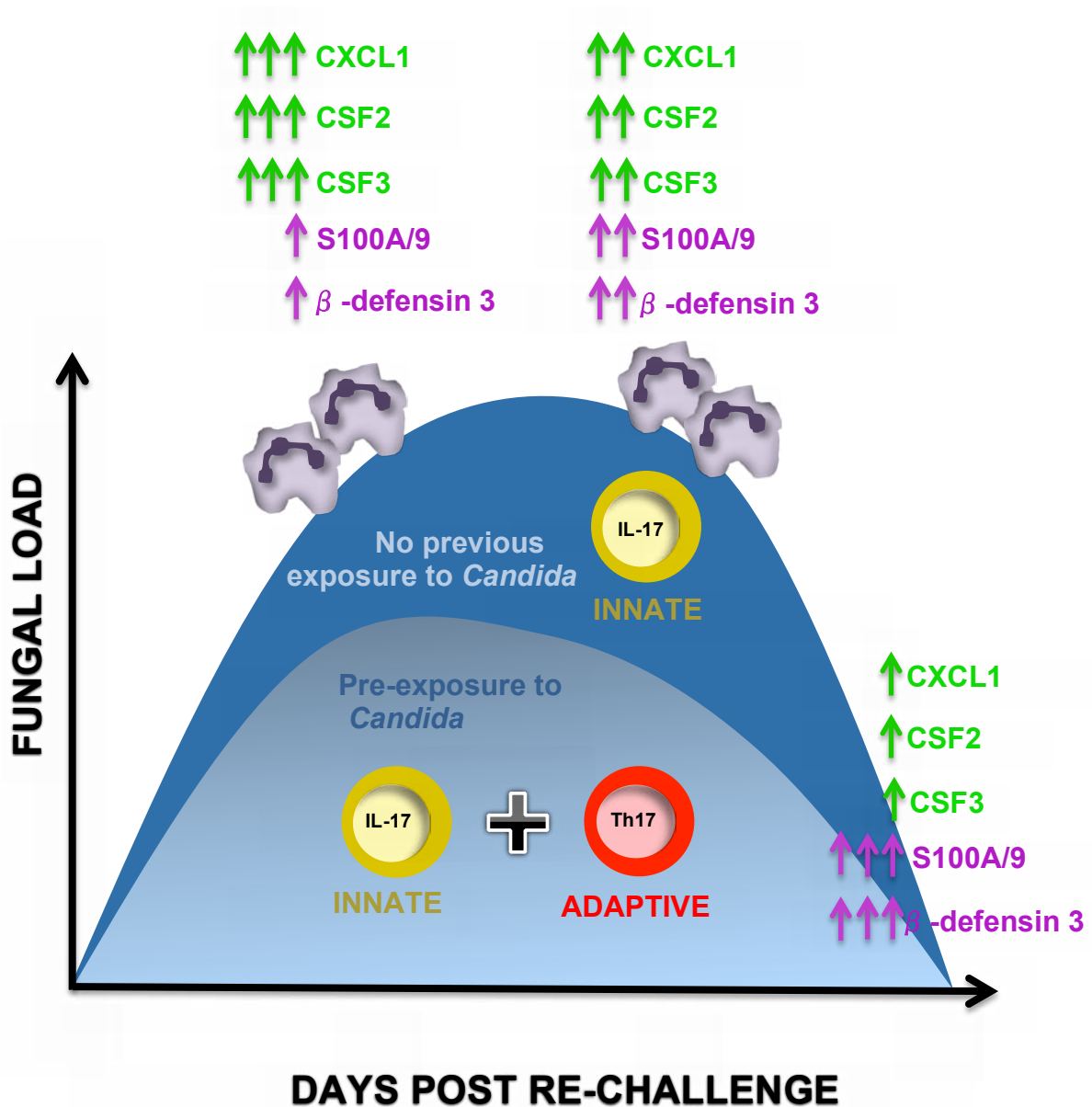
Figure 6.1: Current model of immunity to OPC. In the context of no prior exposure to *C. albicans,* innate immune responses alone can clear infection. Upon prior exposure to *C. albicans,* adaptive immune responses develop and contribute to fungal clearance. The mechanisms of IL-17-mediated immunity employed by both innate and adaptive responses are similar, induction of neutrophil chemotaxis and expression of antimicrobial peptides. Arrows indicate gene expression levels.

6.2 THE ROLE OF INNATE IMMUNITY IN ANTI-*C. ALBICANS* HOST DEFENSE

Our studies demonstrated that innate immunity is an important component of anti-*C. albicans* host defense. Therefore, identifying innate source (s) of IL-17 during OPC is of paramount importance. The fact that Rag1-/- mice are susceptible to OPC indicates that the innate IL-17-producing cell type requires a rearranged receptor. Thus, γδ T cells are interesting candidates because they express a T cell receptor, are abundant in mucosal surfaces, like the gut and the skin, play a role in barrier immunity and express various pattern recognition receptors (Cua and Tato, 2010). In fact, IL-17-producing γδ T cells were shown to express TLR2 and Dectin-1 and to expand in response to *C. albicans* hyphae and curdland (β-glucan) (Martin et al., 2009). In addition, IL-17+ γδ T cells were found in the skin of mice infected with *C. albicans.* The role of γδ T cells in OPC is controversial. An interesting study in nonprogressor HIV+ patients described a subset of circulating memory γδ T cells (Vδ1) that produced IL-17 and IFNγ *ex-vivo* in response to *C. albicans,* suggesting that this subset may compensate for reduced numbers of CD4+ T cells and aid in host responses against opportunistic infections (Fenoglio et al., 2009), such as OPC. However, γδ T cell deficient mice were resistance to OPC.

Another potential candidate as a source of Il-17 is iNKT cells, which express a T cell receptor and have been demonstrated to play an important role during *Aspergillus fumigatus* infection. An elegant study demonstrated that mice deficient in CD1d, an "MHC-like" molecule that presents lipid antigens to iNKT cells, were susceptible to *A. fumigatus* infection (Cohen et al., 2011). Interestingly, iNKT cells also recognized *C. albicans in vitro*, suggesting that iNKT cells may play a role in host responses to

Candida. This study did not address whether or not *in vitro* stimulation with *C. albicans* resulted in IL-17 production by iNKT cells, which is an important question in the context of immunity to OPC. In contrast to what we expected from the aforementioned studies, CD1d-deficient mice were resistant to OPC (Alanna C. Peterson, unpublished).

Given what we know about compensatory mechanisms of IL-17 production, identifying the innate source of IL-17 will require: (1) *in situ* analysis of the immune cell infiltrate by immunofluorescence or immune cell isolation from tongue tissue and subsequent staining of cell surface markers to analyze their expression by flow cytometry. This has proven difficult due to the muscular consistency of the tongue and its high autofluorescence.

In addition to innate sources of IL-17, another concept of innate anti-*Candida* immunity that should be explored in OPC is "trained immunity". A recent study performed in murine disseminated candidiasis demonstrated that Rag1-/- mice, which lack B and T cells, were protected from re-infection with a lethal dose of *C. albicans,* suggesting that an innate cell type had the ability to "memorize" its encounter with *C. albicans* and respond upon re-exposure (Quintin et al., 2012). It was demonstrated that this type of "innate memory" was dependent on the epigenetic reprograming of monocytes, which resulted in increased production of pro-inflammatory cytokines such as IL-6 and TNFα. Our studies demonstrated that, upon pre-exposure to *C. albicans*, the tongue fungal burden is decreased at days 1 and 2 following re-challenge, which is a surprisingly rapid response and, perhaps, suggests the presence of a tissue resident innate population with "memory" properties. Furthermore, protective Th17 cells were not detected in cLN until day 3 after re-challenge. Therefore, the induction of *C.*

albicans-specific Th17 cells does not entirely explain the decrease in fungal burden at days 1 and 2 post re-challenge. The presence of "memory monocytes" during these early time points is an intriguing possibility. In addition, if these monocytes are indeed present during OPC, it will be interesting to study how they influence downstream T cell responses.

6.3   WHERE DO WE GO FROM HERE? THE FUTURE OF ADAPTIVE IMMUNITY TO *CANDIDA ALBICANS*

Recent years have witnessed the development of powerful tools and important discoveries that will allow for more comprehensive studies of antigen-specific responses to *C. albicans.* The engineering of the OVA-expressing *C. albicans* (Calb-Ag) and the discovery of the pALS epitope permit the *in vivo* tracking of T cells during infection (Bar et al., 2012; Igyarto et al., 2011). For example, adoptively transferring OVA-specific T cells into WT mice followed by infection with Calb-Ag will permit detailed dissection of the kinetics of antigen-specific responses in the tongue tissue following infection. Furthermore, labeling of these OVA-specific T cells with tracking dyes, such as CFSE, will be instrumental in imaging studies to determine the location and spatial arrangement of antigen-specific T cells in the tongue. This will provide important information about the interaction of these T cells with other members of the immune system in the oral mucosa, which is largely understudied. In addition, the identification of pALS as a CD4+ T cell epitope opened the door to many possibilities. It is possible to synthesize MHC class II tetramers bearing the pALS peptide, which will be invaluable

for studying *C. albicans*-specific responses *ex-vivo* by flow cytometry. Furthermore, a TCR transgenic mouse where the T cells are pALS-specific will be a much-needed tool in the study of CD4+ T cell responses during *Candida* infections.

Another aspect of anti-*Candida* CD4+ T cell-mediated immunity that disserves careful consideration is the stability of the Th17 phenotype. A significant body of evidence has demonstrated that CD4+ T helper subsets are not fixed entities and rather exhibit considerable "plasticity", which is driven by epigenetic changes and is influenced by the surrounding microenvironment. For example, Tregs can convert to Th17 cells and Th17 cells in turn have the capacity to convert into Th1 and Th2 cells (Hirahara et al., 2011; O'Shea and Paul, 2010). In the context of *C. albicans* infections, *in vitro* studies showed that dendritic cells activated via dectin-1 convert CD25+Foxp3+ Tregs into Foxp3+RORγT+IL-17+ cells in an IL-23-dependent manner (Osorio et al., 2008), suggesting that Tregs aid the pro-inflammatory process and thereby fungal clearance. Accordingly, the co-transfer of *in vitro* polarized Th17 and Treg cells prevented Rag1-/- mice from developing OPC (Pandiyan et al., 2011). However, whether or not *C. albicans* induces Tregs *in vivo* remains to be determined and, if this is the case, do these cells remain as T regs or do they convert to Th17 cells? In addition to the Treg to Th17 conversion, Th17 to Th1 conversion has also been reported in some settings. In fact, memory CD4+ T cells double positive for IL-17 and IFNγ expression were detected following *in vitro* stimulation of human PBMC with *C. albicans* (Zielinski et al., 2012). On the other hand, elegant *in vivo* studies using fate-tracking mice demonstrated that both Th17 cells and γδ+IL-17+ cells induced upon *C. albicans* skin infection exhibited a stable phenotype because no IL-17+IFNγ+ cells or "ex-Th17" cells were detected

(Hirota et al., 2011). This has not been studied in OPC but Th17 to Th1 conversion seems unlikely since we and others have shown that Th1 and IL-17+IFNγ+ cells are absent from the cLN following re-challenge. However, our studies included early time points after re-challenge (Days 1-3) and Th17 to Th1 conversion later during infection is possible.

In light of these observations, it appears that the mechanisms of adaptive immunity to *C. albicans* are only beginning to be unraveled and more detailed studies are needed in order to aid antifungal vaccine design and the effective treatment of fungal infections.

BIBLIOGRAPHY

Acosta-Rodriguez, E.V., L. Rivino, J. Geginat, D. Jarrossay, M. Gattorno, A. Lanzavecchia, F. Sallusto, and G. Napolitani. 2007. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol* 8:639-646.

Akpan, A., and R. Morgan. 2002. Oral candidiasis. *Postgrad Med J* 78:455-459.

Ashman, R.B., J.M. Papadimitriou, A. Fulurija, K.E. Drysdale, C.S. Farah, O. Naidoo, and T. Gotjamanos. 2003. Role of complement C5 and T lymphocytes in pathogenesis of disseminated and mucosal candidiasis in susceptible DBA/2 mice. *Microb Pathog* 34:103-113.

Bar, E., A. Gladiator, S. Bastidas, B. Roschitzki, H. Acha-Orbea, A. Oxenius, and S. LeibundGut-Landmann. 2012. A novel Th cell epitope of Candida albicans mediates protection from fungal infection. *J Immunol* 188:5636-5643.

Bellocchio, S., C. Montagnoli, S. Bozza, R. Gaziano, G. Rossi, S.S. Mambula, A. Vecchi, A. Mantovani, S.M. Levitz, and L. Romani. 2004. The contribution of the Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens in vivo. *J Immunol* 172:3059-3069.

Bi, L., S. Gojestani, W. Wu, Y.M. Hsu, J. Zhu, K. Ariizumi, and X. Lin. 2010. CARD9 mediates dectin-2-induced IkappaBalpha kinase ubiquitination leading to activation of NF-kappaB in response to stimulation by the hyphal form of Candida albicans. *J Biol Chem* 285:25969-25977.

Bozza, S., C. Montagnoli, R. Gaziano, G. Rossi, G. Nkwanyuo, S. Bellocchio, and L. Romani. 2004. Dendritic cell-based vaccination against opportunistic fungi. *Vaccine* 22:857-864.

Brown, G.D. 2010. How fungi have shaped our understanding of mammalian immunology. *Cell Host Microbe* 7:9-11.

Buonocore, S., P.P. Ahern, H.H. Uhlig, Ivanov, II, D.R. Littman, K.J. Maloy, and F. Powrie. 2010. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. *Nature* 464:1371-1375.

Cambi, A., M.G. Netea, H.M. Mora-Montes, N.A. Gow, S.V. Hato, D.W. Lowman, B.J. Kullberg, R. Torensma, D.L. Williams, and C.G. Figdor. 2008. Dendritic cell

interaction with Candida albicans critically depends on N-linked mannan. *J Biol Chem* 283:20590-20599.

Casadevall, A., and L.A. Pirofski. 2012. Immunoglobulins in defense, pathogenesis, and therapy of fungal diseases. *Cell Host Microbe* 11:447-456.

Cella, M., A. Fuchs, W. Vermi, F. Facchetti, K. Otero, J.K. Lennerz, J.M. Doherty, J.C. Mills, and M. Colonna. 2009. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature* 457:722-725.

Cenci, E., A. Mencacci, G. Del Sero, C.F. d'Ostiani, P. Mosci, A. Bacci, C. Montagnoli, M. Kopf, and L. Romani. 1998. IFN-gamma is required for IL-12 responsiveness in mice with Candida albicans infection. *J Immunol* 161:3543-3550.

Cenci, E., A. Mencacci, R. Spaccapelo, L. Tonnetti, P. Mosci, K.H. Enssle, P. Puccetti, L. Romani, and F. Bistoni. 1995. T helper cell type 1 (Th1)- and Th2-like responses are present in mice with gastric candidiasis but protective immunity is associated with Th1 development. *J Infect Dis* 171:1279-1288.

Chen, Z., A. Laurence, Y. Kanno, M. Pacher-Zavisin, B.M. Zhu, C. Tato, A. Yoshimura, L. Hennighausen, and J.J. O'Shea. 2006. Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. *Proc Natl Acad Sci U S A* 103:8137-8142.

Chen, Z.M., and M.K. Jenkins. 1998. Revealing the in vivo behavior of CD4+ T cells specific for an antigen expressed in Escherichia coli. *J Immunol* 160:3462-3470.

Cheng, S.C., F. van de Veerdonk, S. Smeekens, L.A. Joosten, J.W. van der Meer, B.J. Kullberg, and M.G. Netea. 2010. Candida albicans dampens host defense by downregulating IL-17 production. *J Immunol* 185:2450-2457.

Cheng, S.C., F.L. van de Veerdonk, M. Lenardon, M. Stoffels, T. Plantinga, S. Smeekens, L. Rizzetto, L. Mukaremera, K. Preechasuth, D. Cavalieri, T.D. Kanneganti, J.W. van der Meer, B.J. Kullberg, L.A. Joosten, N.A. Gow, and M.G. Netea. 2011. The dectin-1/inflammasome pathway is responsible for the induction of protective T-helper 17 responses that discriminate between yeasts and hyphae of Candida albicans. *J Leukoc Biol* 90:357-366.

Cohen, N.R., R.V. Tatituri, A. Rivera, G.F. Watts, E.Y. Kim, A. Chiba, B.B. Fuchs, E. Mylonakis, G.S. Besra, S.M. Levitz, M. Brigl, and M.B. Brenner. 2011. Innate recognition of cell wall beta-glucans drives invariant natural killer T cell responses against fungi. *Cell Host Microbe* 10:437-450.

Colonna, M. 2009. Interleukin-22-producing natural killer cells and lymphoid tissue inducer-like cells in mucosal immunity. *Immunity* 31:15-23.

Conti, H.R., O. Baker, A.F. Freeman, W.S. Jang, S.M. Holland, R.A. Li, M. Edgerton, and S.L. Gaffen. 2011. New mechanism of oral immunity to mucosal candidiasis in hyper-IgE syndrome. *Mucosal Immunol* 4:448-455.

Conti, H.R., and S.L. Gaffen. 2010. Host responses to Candida albicans: Th17 cells and mucosal candidiasis. *Microbes Infect* 12:518-527.

Conti, H.R., F. Shen, N. Nayyar, E. Stocum, J.N. Sun, M.J. Lindemann, A.W. Ho, J.H. Hai, J.J. Yu, J.W. Jung, S.G. Filler, P. Masso-Welch, M. Edgerton, and S.L. Gaffen. 2009. Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. *J Exp Med* 206:299-311.

Crellin, N.K., S. Trifari, C.D. Kaplan, T. Cupedo, and H. Spits. 2010. Human NKp44+IL-22+ cells and LTi-like cells constitute a stable RORC+ lineage distinct from conventional natural killer cells. *The Journal of Experimental Medicine* 207:281-290.

Cua, D.J., and C.M. Tato. 2010. Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol* 10:479-489.

Cupedo, T., N.K. Crellin, N. Papazian, E.J. Rombouts, K. Weijer, J.L. Grogan, W.E. Fibbe, J.J. Cornelissen, and H. Spits. 2009. Human fetal lymphoid tissue-inducer cells are interleukin 17-producing precursors to RORC+ CD127+ natural killer-like cells. *Nat Immunol* 10:66-74.

Cutler, C.W., and R. Jotwani. 2006. Dendritic cells at the oral mucosal interface. *J Dent Res* 85:678-689.

De Luca, A., T. Zelante, C. D'Angelo, S. Zagarella, F. Fallarino, A. Spreca, R.G. Iannitti, P. Bonifazi, J.C. Renauld, F. Bistoni, P. Puccetti, and L. Romani. 2010. IL-22 defines a novel immune pathway of antifungal resistance. *Mucosal Immunol* 3:361-373.

Del Favero, A. 2000. Management of fungal infections in neutropenic patients: more doubts than certainties? *Int J Antimicrob Agents* 16:135-137.

Doisne, J.M., C. Becourt, L. Amniai, N. Duarte, J.B. Le Luduec, G. Eberl, and K. Benlagha. 2009. Skin and peripheral lymph node invariant NKT cells are mainly retinoic acid receptor-related orphan receptor (gamma)t+ and respond preferentially under inflammatory conditions. *J Immunol* 183:2142-2149.

Dongari-Bagtzoglou, A., and H. Kashleva. 2003. Candida albicans triggers interleukin-8 secretion by oral epithelial cells. *Microb Pathog* 34:169-177.

Dongari-Bagtzoglou, A., C.C. Villar, and H. Kashleva. 2005. Candida albicans-infected oral epithelial cells augment the anti-fungal activity of human neutrophils in vitro. *Med Mycol* 43:545-549.

Esteban, A., M.W. Popp, V.K. Vyas, K. Strijbis, H.L. Ploegh, and G.R. Fink. 2011. Fungal recognition is mediated by the association of dectin-1 and galectin-3 in macrophages. *Proc Natl Acad Sci U S A* 108:14270-14275.

Eyerich, S., J. Wagener, V. Wenzel, C. Scarponi, D. Pennino, C. Albanesi, M. Schaller, H. Behrendt, J. Ring, C.B. Schmidt-Weber, A. Cavani, M. Mempel, C. Traidl-Hoffmann, and K. Eyerich. 2011. IL-22 and TNF-alpha represent a key cytokine combination for epidermal integrity during infection with Candida albicans. *Eur J Immunol* 41:1894-1901.

Farah, C.S., S. Elahi, K. Drysdale, G. Pang, T. Gotjamanos, G.J. Seymour, R.L. Clancy, and R.B. Ashman. 2002a. Primary role for CD4(+) T lymphocytes in recovery from oropharyngeal candidiasis. *Infect Immun* 70:724-731.

Farah, C.S., T. Gotjamanos, G.J. Seymour, and R.B. Ashman. 2002b. Cytokines in the oral mucosa of mice infected with Candida albicans. *Oral Microbiol Immunol* 17:375-378.

Farah, C.S., Y. Hu, S. Riminton, and R.B. Ashman. 2006. Distinct roles for interleukin-12p40 and tumour necrosis factor in resistance to oral candidiasis defined by gene-targeting. *Oral Microbiol Immunol* 21:252-255.

Fenoglio, D., A. Poggi, S. Catellani, F. Battaglia, A. Ferrera, M. Setti, G. Murdaca, and M.R. Zocchi. 2009. Vdelta1 T lymphocytes producing IFN-gamma and IL-17 are expanded in HIV-1-infected patients and respond to Candida albicans. *Blood* 113:6611-6618.

Ferretti, S., O. Bonneau, G.R. Dubois, C.E. Jones, and A. Trifilieff. 2003. IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: IL-15 as a possible trigger. *J Immunol* 170:2106-2112.

Ferwerda, B., G. Ferwerda, T.S. Plantinga, J.A. Willment, A.B. van Spriel, H. Venselaar, C.C. Elbers, M.D. Johnson, A. Cambi, C. Huysamen, L. Jacobs, T. Jansen, K. Verheijen, L. Masthoff, S.A. Morre, G. Vriend, D.L. Williams, J.R. Perfect, L.A. Joosten, C. Wijmenga, J.W. van der Meer, G.J. Adema, B.J. Kullberg, G.D. Brown, and M.G. Netea. 2009. Human dectin-1 deficiency and mucocutaneous fungal infections. *N Engl J Med* 361:1760-1767.

Fidel, P.L., Jr. 2011. Candida-host interactions in HIV disease: implications for oropharyngeal candidiasis. *Adv Dent Res* 23:45-49.

Filler, S.G. 2012. Insights from human studies into the host defense against candidiasis. *Cytokine* 58:129-132.

Gaffen, S.L. 2009. Structure and signalling in the IL-17 receptor family. *Nat Rev Immunol* 9:556-567.

Ghilardi, N., and W. Ouyang. 2007. Targeting the development and effector functions of TH17 cells. *Semin Immunol* 19:383-393.

Ghoreschi, K., A. Laurence, X.P. Yang, C.M. Tato, M.J. McGeachy, J.E. Konkel, H.L. Ramos, L. Wei, T.S. Davidson, N. Bouladoux, J.R. Grainger, Q. Chen, Y. Kanno, W.T. Watford, H.W. Sun, G. Eberl, E.M. Shevach, Y. Belkaid, D.J. Cua, W. Chen, and J.J. O'Shea. 2010. Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling. *Nature* 467:967-971.

Glocker, E.O., A. Hennigs, M. Nabavi, A.A. Schaffer, C. Woellner, U. Salzer, D. Pfeifer, H. Veelken, K. Warnatz, F. Tahami, S. Jamal, A. Manguiat, N. Rezaei, A.A. Amirzargar, A. Plebani, N. Hannesschlager, O. Gross, J. Ruland, and B. Grimbacher. 2009. A homozygous CARD9 mutation in a family with susceptibility to fungal infections. *N Engl J Med* 361:1727-1735.

Gorr, S.U. 2009. Antimicrobial peptides of the oral cavity. *Periodontol 2000* 51:152-180.

Gow, N.A.R. 2012. Candida albicans morphogenesis and host defence: discriminating invasion from colonization. *Nat. Rev. Microbiol* 10:

Grigull, L., A. Beilken, H. Schmid, P. Kirschner, K.W. Sykora, C. Linderkamp, F. Donnerstag, L. Goudeva, H.G. Heuft, and K. Welte. 2006. Secondary prophylaxis of invasive fungal infections with combination antifungal therapy and G-CSF-mobilized granulocyte transfusions in three children with hematological malignancies. *Support Care Cancer* 14:783-786.

Gringhuis, S.I., T.M. Kaptein, B.A. Wevers, B. Theelen, M. van der Vlist, T. Boekhout, and T.B. Geijtenbeek. 2012. Dectin-1 is an extracellular pathogen sensor for the induction and processing of IL-1beta via a noncanonical caspase-8 inflammasome. *Nat Immunol* 13:246-254.

Gross, O., H. Poeck, M. Bscheider, C. Dostert, N. Hannesschlager, S. Endres, G. Hartmann, A. Tardivel, E. Schweighoffer, V. Tybulewicz, A. Mocsai, J. Tschopp, and J. Ruland. 2009. Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature* 459:433-436.

Harrington, L.E., R.D. Hatton, P.R. Mangan, H. Turner, T.L. Murphy, K.M. Murphy, and C.T. Weaver. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 6:1123-1132.

Hayday, A.C. 2009. Gammadelta T cells and the lymphoid stress-surveillance response. *Immunity* 31:184-196.

Hirahara, K., G. Vahedi, K. Ghoreschi, X.P. Yang, S. Nakayamada, Y. Kanno, J.J. O'Shea, and A. Laurence. 2011. Helper T-cell differentiation and plasticity: insights from epigenetics. *Immunology* 134:235-245.

Hirota, K., J.H. Duarte, M. Veldhoen, E. Hornsby, Y. Li, D.J. Cua, H. Ahlfors, C. Wilhelm, M. Tolaini, U. Menzel, A. Garefalaki, A.J. Potocnik, and B. Stockinger. 2011. Fate mapping of IL-17-producing T cells in inflammatory responses. *Nat Immunol* 12:255-263.

Hise, A.G., J. Tomalka, S. Ganesan, K. Patel, B.A. Hall, G.D. Brown, and K.A. Fitzgerald. 2009. An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen Candida albicans. *Cell Host Microbe* 5:487-497.

Ho, A.W., F. Shen, H.R. Conti, N. Patel, E.E. Childs, A.C. Peterson, N. Hernandez-Santos, J.K. Kolls, L.P. Kane, W. Ouyang, and S.L. Gaffen. 2010. IL-17RC is required for immune signaling via an extended SEF/IL-17R signaling domain in the cytoplasmic tail. *J Immunol* 185:1063-1070.

Huang, W., L. Na, P.L. Fidel, and P. Schwarzenberger. 2004. Requirement of interleukin-17A for systemic anti-Candida albicans host defense in mice. *J Infect Dis* 190:624-631.

Igyarto, B.Z., K. Haley, D. Ortner, A. Bobr, M. Gerami-Nejad, B.T. Edelson, S.M. Zurawski, B. Malissen, G. Zurawski, J. Berman, and D.H. Kaplan. 2011. Skin-resident murine dendritic cell subsets promote distinct and opposing antigen-specific T helper cell responses. *Immunity* 35:260-272.

Ishikawa, E., T. Ishikawa, Y.S. Morita, K. Toyonaga, H. Yamada, O. Takeuchi, T. Kinoshita, S. Akira, Y. Yoshikai, and S. Yamasaki. 2009. Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. *The Journal of Experimental Medicine* 206:2879-2888.

Iwasaki, A., and R. Medzhitov. 2004. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 5:987-995.

Joly, S., S.C. Eisenbarth, A.K. Olivier, A. Williams, D.H. Kaplan, S.L. Cassel, R.A. Flavell, and F.S. Sutterwala. 2012. Cutting Edge: Nlrp10 Is Essential for Protective Antifungal Adaptive Immunity against Candida albicans. *J Immunol* 189:4713-4717.

Kagami, S., H.L. Rizzo, S.E. Kurtz, L.S. Miller, and A. Blauvelt. 2010. IL-23 and IL-17A, but not IL-12 and IL-22, are required for optimal skin host defense against Candida albicans. *J Immunol* 185:5453-5462.

Khader, S.A., S.L. Gaffen, and J.K. Kolls. 2009. Th17 cells at the crossroads of innate and adaptive immunity against infectious diseases at the mucosa. *Mucosal Immunol* 2:403-411.

Kisand, K., A.S. Boe Wolff, K.T. Podkrajsek, L. Tserel, M. Link, K.V. Kisand, E. Ersvaer, J. Perheentupa, M.M. Erichsen, N. Bratanic, A. Meloni, F. Cetani, R. Perniola, B. Ergun-Longmire, N. Maclaren, K.J. Krohn, M. Pura, B. Schalke, P. Strobel, M.I.

Leite, T. Battelino, E.S. Husebye, P. Peterson, N. Willcox, and A. Meager. 2010. Chronic mucocutaneous candidiasis in APECED or thymoma patients correlates with autoimmunity to Th17-associated cytokines. *The Journal of Experimental Medicine* 207:299-308.

Laurence, A., C.M. Tato, T.S. Davidson, Y. Kanno, Z. Chen, Z. Yao, R.B. Blank, F. Meylan, R. Siegel, L. Hennighausen, E.M. Shevach, and J. O'Shea J. 2007. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity* 26:371-381.

Lee, S.J. 2003. Normal Host Defense during Systemic Candidiasis in Mannose Receptor-Deficient Mice. *Infection and Immunity* 71:

Lee, Y., A. Awasthi, N. Yosef, F.J. Quintana, S. Xiao, A. Peters, C. Wu, M. Kleinewietfeld, S. Kunder, D.A. Hafler, R.A. Sobel, A. Regev, and V.K. Kuchroo. 2012. Induction and molecular signature of pathogenic TH17 cells. *Nat Immunol* 13:991-999.

LeibundGut-Landmann, S., O. Gross, M.J. Robinson, F. Osorio, E.C. Slack, S.V. Tsoni, E. Schweighoffer, V. Tybulewicz, G.D. Brown, J. Ruland, and C. Reis e Sousa. 2007. Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. *Nat Immunol* 8:630-638.

Li, L., L. Huang, A.L. Vergis, H. Ye, A. Bajwa, V. Narayan, R.M. Strieter, D.L. Rosin, and M.D. Okusa. 2010. IL-17 produced by neutrophils regulates IFN-gamma-mediated neutrophil migration in mouse kidney ischemia-reperfusion injury. *The Journal of Clinical Investigation* 120:331-342.

Liang, S.C., X.Y. Tan, D.P. Luxenberg, R. Karim, K. Dunussi-Joannopoulos, M. Collins, and L.A. Fouser. 2006. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *The Journal of Experimental Medicine* 203:2271-2279.

Lin, L. 2009. Th1-Th17 Cells Mediate Protective Adaptive Immunity against *Staphylococcus aureus* and *Candida albicans* Infection in Mice. *PLoS Pathogens* 5:

Liu, L., S. Okada, X.F. Kong, A.Y. Kreins, S. Cypowyj, A. Abhyankar, J. Toubiana, Y. Itan, M. Audry, P. Nitschke, C. Masson, B. Toth, J. Flatot, M. Migaud, M. Chrabieh, T. Kochetkov, A. Bolze, A. Borghesi, A. Toulon, J. Hiller, S. Eyerich, K. Eyerich, V. Gulacsy, L. Chernyshova, V. Chernyshov, A. Bondarenko, R.M. Grimaldo, L. Blancas-Galicia, I.M. Beas, J. Roesler, K. Magdorf, D. Engelhard, C. Thumerelle, P.R. Burgel, M. Hoernes, B. Drexel, R. Seger, T. Kusuma, A.F. Jansson, J. Sawalle-Belohradsky, B. Belohradsky, E. Jouanguy, J. Bustamante, M. Bue, N. Karin, G. Wildbaum, C. Bodemer, O. Lortholary, A. Fischer, S. Blanche, S. Al-Muhsen, J. Reichenbach, M. Kobayashi, F.E. Rosales, C.T. Lozano, S.S. Kilic, M. Oleastro, A. Etzioni, C. Traidl-Hoffmann, E.D. Renner, L. Abel, C. Picard, L. Marodi, S. Boisson-Dupuis, A. Puel, and J.L. Casanova. 2011.

Gain-of-function human STAT1 mutations impair IL-17 immunity and underlie chronic mucocutaneous candidiasis. *The Journal of Experimental Medicine* 208:1635-1648.

Lochner, M., L. Peduto, M. Cherrier, S. Sawa, F. Langa, R. Varona, D. Riethmacher, M. Si-Tahar, J.P. Di Santo, and G. Eberl. 2008. In vivo equilibrium of proinflammatory IL-17+ and regulatory IL-10+ Foxp3+ RORgamma t+ T cells. *The Journal of Experimental Medicine* 205:1381-1393.

Luci, C., A. Reynders, Ivanov, II, C. Cognet, L. Chiche, L. Chasson, J. Hardwigsen, E. Anguiano, J. Banchereau, D. Chaussabel, M. Dalod, D.R. Littman, E. Vivier, and E. Tomasello. 2009. Influence of the transcription factor RORgammat on the development of NKp46+ cell populations in gut and skin. *Nat Immunol* 10:75-82.

Ma, C.S., G.Y. Chew, N. Simpson, A. Priyadarshi, M. Wong, B. Grimbacher, D.A. Fulcher, S.G. Tangye, and M.C. Cook. 2008. Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. *J Exp Med* 205:1551-1557.

Maertens, J., M. Vrebos, and M. Boogaerts. 2001. Assessing risk factors for systemic fungal infections. *Eur J Cancer Care (Engl)* 10:56-62.

Martin, B., K. Hirota, D.J. Cua, B. Stockinger, and M. Veldhoen. 2009. Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. *Immunity* 31:321-330.

Massey, S.E., G. Moura, P. Beltrao, R. Almeida, J.R. Garey, M.F. Tuite, and M.A. Santos. 2003. Comparative evolutionary genomics unveils the molecular mechanism of reassignment of the CTG codon in Candida spp. *Genome Res* 13:544-557.

McGeachy, M.J., Y. Chen, C.M. Tato, A. Laurence, B. Joyce-Shaikh, W.M. Blumenschein, T.K. McClanahan, J.J. O'Shea, and D.J. Cua. 2009. The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo. *Nat Immunol* 10:314-324.

McNulty, K.M., J. Plianrungsi, J.E. Leigh, D. Mercante, and P.L. Fidel, Jr. 2005. Characterization of CD8+ T cells and microenvironment in oral lesions of human immunodeficiency virus-infected persons with oropharyngeal candidiasis. *Infection and Immunity* 73:3659-3667.

Michel, M.L., D. Mendes-da-Cruz, A.C. Keller, M. Lochner, E. Schneider, M. Dy, G. Eberl, and M.C. Leite-de-Moraes. 2008. Critical role of ROR-gammat in a new thymic pathway leading to IL-17-producing invariant NKT cell differentiation. *Proc Natl Acad Sci U S A* 105:19845-19850.

Milner, J.D., J.M. Brenchley, A. Laurence, A.F. Freeman, B.J. Hill, K.M. Elias, Y. Kanno, C. Spalding, H.Z. Elloumi, M.L. Paulson, J. Davis, A. Hsu, A.I. Asher, J. O'Shea, S.M. Holland, W.E. Paul, and D.C. Douek. 2008. Impaired TH17 cell

differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature* 452:773-776.

Minegishi, Y., M. Saito, S. Tsuchiya, I. Tsuge, H. Takada, T. Hara, N. Kawamura, T. Ariga, S. Pasic, O. Stojkovic, A. Metin, and H. Karasuyama. 2007. Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome. *Nature* 448:1058-1062.

Muranski, P., Z.A. Borman, S.P. Kerkar, C.A. Klebanoff, Y. Ji, L. Sanchez-Perez, M. Sukumar, R.N. Reger, Z. Yu, S.J. Kern, R. Roychoudhuri, G.A. Ferreyra, W. Shen, S.K. Durum, L. Feigenbaum, D.C. Palmer, P.A. Antony, C.C. Chan, A. Laurence, R.L. Danner, L. Gattinoni, and N.P. Restifo. 2011. Th17 cells are long lived and retain a stem cell-like molecular signature. *Immunity* 35:972-985.

Myers, T.A., J.E. Leigh, A.R. Arribas, S. Hager, R. Clark, E. Lilly, and P.L. Fidel, Jr. 2003. Immunohistochemical evaluation of T cells in oral lesions from human immunodeficiency virus-positive persons with oropharyngeal candidiasis. *Infection and Immunity* 71:956-963.

Nanjappa, S.G., E. Heninger, M. Wuthrich, D.J. Gasper, and B.S. Klein. 2012a. Tc17 cells mediate vaccine immunity against lethal fungal pneumonia in immune deficient hosts lacking CD4+ T cells. *PLoS Pathogens* 8:e1002771.

Nanjappa, S.G., E. Heninger, M. Wuthrich, T. Sullivan, and B. Klein. 2012b. Protective antifungal memory CD8(+) T cells are maintained in the absence of CD4(+) T cell help and cognate antigen in mice. *The Journal of Clinical Investigation* 122:987-999.

Netea, M.G. 2006. Immune sensing of Candida albicans requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. *The Journal of Clinical Investigation* 116:

Netea, M.G., G.D. Brown, B.J. Kullberg, and N.A. Gow. 2008. An integrated model of the recognition of Candida albicans by the innate immune system. *Nat Rev Microbiol* 6:67-78.

Netea, M.G., R. Sutmuller, C. Hermann, C.A. Van der Graaf, J.W. Van der Meer, J.H. van Krieken, T. Hartung, G. Adema, and B.J. Kullberg. 2004. Toll-like receptor 2 suppresses immunity against Candida albicans through induction of IL-10 and regulatory T cells. *J Immunol* 172:3712-3718.

Netea, M.G., C.A. Van Der Graaf, A.G. Vonk, I. Verschueren, J.W. Van Der Meer, and B.J. Kullberg. 2002. The role of toll-like receptor (TLR) 2 and TLR4 in the host defense against disseminated candidiasis. *J Infect Dis* 185:1483-1489.

Nigam, P., S. Kwa, V. Velu, and R.R. Amara. 2011. Loss of IL-17-producing CD8 T cells during late chronic stage of pathogenic simian immunodeficiency virus infection. *J Immunol* 186:745-753.

Nobile, C.J., H.A. Schneider, J.E. Nett, D.C. Sheppard, S.G. Filler, D.R. Andes, and A.P. Mitchell. 2008. Complementary adhesin function in C. albicans biofilm formation. *Curr Biol* 18:1017-1024.

O'Shea, J.J., and W.E. Paul. 2010. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science* 327:1098-1102.

Osorio, F., S. LeibundGut-Landmann, M. Lochner, K. Lahl, T. Sparwasser, G. Eberl, and C. Reis e Sousa. 2008. DC activated via dectin-1 convert Treg into IL-17 producers. *Eur J Immunol* 38:3274-3281.

Pandiyan, P., H.R. Conti, L. Zheng, A.C. Peterson, D.R. Mathern, N. Hernandez-Santos, M. Edgerton, S.L. Gaffen, and M.J. Lenardo. 2011. CD4(+)CD25(+)Foxp3(+) regulatory T cells promote Th17 cells in vitro and enhance host resistance in mouse Candida albicans Th17 cell infection model. *Immunity* 34:422-434.

Pepper, M., J.L. Linehan, A.J. Pagan, T. Zell, T. Dileepan, P.P. Cleary, and M.K. Jenkins. 2010. Different routes of bacterial infection induce long-lived TH1 memory cells and short-lived TH17 cells. *Nat Immunol* 11:83-89.

Pietrella, D., A. Rachini, M. Pines, N. Pandey, P. Mosci, F. Bistoni, C. d'Enfert, and A. Vecchiarelli. 2011. Th17 cells and IL-17 in protective immunity to vaginal candidiasis. *PLoS One* 6:e22770.

Plantinga, T.S., W.J. van der Velden, B. Ferwerda, A.B. van Spriel, G. Adema, T. Feuth, J.P. Donnelly, G.D. Brown, B.J. Kullberg, N.M. Blijlevens, and M.G. Netea. 2009. Early stop polymorphism in human DECTIN-1 is associated with increased candida colonization in hematopoietic stem cell transplant recipients. *Clin Infect Dis* 49:724-732.

Puel, A. 2011. Chronic Mucocutaneous Candidiasis in Humans with Inborn Errors of Interleukin-17 Immunity. *Science*

Puel, A., R. Doffinger, A. Natividad, M. Chrabieh, G. Barcenas-Morales, C. Picard, A. Cobat, M. Ouachee-Chardin, A. Toulon, J. Bustamante, S. Al-Muhsen, M. Al-Owain, P.D. Arkwright, C. Costigan, V. McConnell, A.J. Cant, M. Abinun, M. Polak, P.F. Bougneres, D. Kumararatne, L. Marodi, A. Nahum, C. Roifman, S. Blanche, A. Fischer, C. Bodemer, L. Abel, D. Lilic, and J.L. Casanova. Autoantibodies against IL-17A, IL-17F, and IL-22 in patients with chronic mucocutaneous candidiasis and autoimmune polyendocrine syndrome type I. *J Exp Med* 207:291-297.

Puel, A., R. Doffinger, A. Natividad, M. Chrabieh, G. Barcenas-Morales, C. Picard, A. Cobat, M. Ouachee-Chardin, A. Toulon, J. Bustamante, S. Al-Muhsen, M. Al-Owain, P.D. Arkwright, C. Costigan, V. McConnell, A.J. Cant, M. Abinun, M. Polak, P.F. Bougneres, D. Kumararatne, L. Marodi, A. Nahum, C. Roifman, S. Blanche, A. Fischer, C. Bodemer, L. Abel, D. Lilic, and J.L. Casanova. 2010.

Autoantibodies against IL-17A, IL-17F, and IL-22 in patients with chronic mucocutaneous candidiasis and autoimmune polyendocrine syndrome type I. *The Journal of Experimental Medicine* 207:291-297.

Quintin, J., S. Saeed, J.H. Martens, E.J. Giamarellos-Bourboulis, D.C. Ifrim, C. Logie, L. Jacobs, T. Jansen, B.J. Kullberg, C. Wijmenga, L.A. Joosten, R.J. Xavier, J.W. van der Meer, H.G. Stunnenberg, and M.G. Netea. 2012. Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. *Cell Host Microbe* 12:223-232.

Rivera, A. 2011. Dectin-1 diversifies Aspergillus fumigatus– specific T cell responses by inhibiting T helper type 1 CD4 T cell differentiation. *The Journal of Experimental Medicine*

Robinson, M.J., F. Osorio, M. Rosas, R.P. Freitas, E. Schweighoffer, O. Gross, J.S. Verbeek, J. Ruland, V. Tybulewicz, G.D. Brown, L.F. Moita, P.R. Taylor, and C. Reis e Sousa. 2009. Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. *J Exp Med* 206:2037-2051.

Romani, L. 2011. Immunity to Fungal Infections. *Nat. Rev. Immunol.* 11:

Saijo, S., N. Fujikado, T. Furuta, S.H. Chung, H. Kotaki, K. Seki, K. Sudo, S. Akira, Y. Adachi, N. Ohno, T. Kinjo, K. Nakamura, K. Kawakami, and Y. Iwakura. 2007. Dectin-1 is required for host defense against Pneumocystis carinii but not against Candida albicans. *Nat Immunol* 8:39-46.

Saijo, S., S. Ikeda, K. Yamabe, S. Kakuta, H. Ishigame, A. Akitsu, N. Fujikado, T. Kusaka, S. Kubo, S.H. Chung, R. Komatsu, N. Miura, Y. Adachi, N. Ohno, K. Shibuya, N. Yamamoto, K. Kawakami, S. Yamasaki, T. Saito, S. Akira, and Y. Iwakura. 2010. Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against Candida albicans. *Immunity* 32:681-691.

Saijo, S., and Y. Iwakura. 2011. Dectin-1 and Dectin-2 in innate immunity against fungi. *Int Immunol* 23:467-472.

Sanos, S.L., and A. Diefenbach. 2010. Isolation of NK cells and NK-like cells from the intestinal lamina propria. *Methods Mol Biol* 612:505-517.

Satoh-Takayama, N., S. Lesjean-Pottier, P. Vieira, S. Sawa, G. Eberl, C.A. Vosshenrich, and J.P. Di Santo. 2010. IL-7 and IL-15 independently program the differentiation of intestinal CD3-NKp46+ cell subsets from Id2-dependent precursors. *The Journal of Experimental Medicine* 207:273-280.

Satoh-Takayama, N., C.A. Vosshenrich, S. Lesjean-Pottier, S. Sawa, M. Lochner, F. Rattis, J.J. Mention, K. Thiam, N. Cerf-Bensussan, O. Mandelboim, G. Eberl, and J.P. Di Santo. 2008. Microbial flora drives interleukin 22 production in intestinal

NKp46+ cells that provide innate mucosal immune defense. *Immunity* 29:958-970.

Schoenen, H., B. Bodendorfer, K. Hitchens, S. Manzanero, K. Werninghaus, F. Nimmerjahn, E.M. Agger, S. Stenger, P. Andersen, J. Ruland, G.D. Brown, C. Wells, and R. Lang. 2010. Cutting edge: Mincle is essential for recognition and adjuvanticity of the mycobacterial cord factor and its synthetic analog trehalose-dibehenate. *J Immunol* 184:2756-2760.

Scurlock, A.M., L.C. Frazer, C.W. Andrews, Jr., C.M. O'Connell, I.P. Foote, S.L. Bailey, K. Chandra-Kuntal, J.K. Kolls, and T. Darville. 2011. Interleukin-17 contributes to generation of Th1 immunity and neutrophil recruitment during Chlamydia muridarum genital tract infection but is not required for macrophage influx or normal resolution of infection. *Infection and Immunity* 79:1349-1362.

Sieck, T.G., M.A. Moors, H.R. Buckley, and K.J. Blank. 1993. Protection against murine disseminated candidiasis mediated by a Candida albicans-specific T-cell line. *Infect Immun* 61:3540-3543.

Spellberg, B.J., A.S. Ibrahim, V. Avanesian, Y. Fu, C. Myers, Q.T. Phan, S.G. Filler, M.R. Yeaman, and J.E. Edwards, Jr. 2006. Efficacy of the anti-Candida rAls3p-N or rAls1p-N vaccines against disseminated and mucosal candidiasis. *J Infect Dis* 194:256-260.

Strasser, D., K. Neumann, H. Bergmann, M.J. Marakalala, R. Guler, A. Rojowska, K.P. Hopfner, F. Brombacher, H. Urlaub, G. Baier, G.D. Brown, M. Leitges, and J. Ruland. 2012. Syk kinase-coupled C-type lectin receptors engage protein kinase C-sigma to elicit Card9 adaptor-mediated innate immunity. *Immunity* 36:32-42.

Sutton, C.E., S.J. Lalor, C.M. Sweeney, C.F. Brereton, E.C. Lavelle, and K.H. Mills. 2009. Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity* 31:331-341.

Takahara, K., S. Tokieda, K. Nagaoka, and K. Inaba. 2012. Efficient capture of Candida albicans and zymosan by SIGNR1 augments TLR2-dependent TNF-alpha production. *Int Immunol* 24:89-96.

Takahashi, N., I. Vanlaere, R. de Rycke, A. Cauwels, L.A. Joosten, E. Lubberts, W.B. van den Berg, and C. Libert. 2008. IL-17 produced by Paneth cells drives TNF-induced shock. *The Journal of Experimental Medicine* 205:1755-1761.

Takatori, H., Y. Kanno, W.T. Watford, C.M. Tato, G. Weiss, Ivanov, II, D.R. Littman, and J.J. O'Shea. 2009. Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. *The Journal of Experimental Medicine* 206:35-41.

Taylor, P.R., S.V. Tsoni, J.A. Willment, K.M. Dennehy, M. Rosas, H. Findon, K. Haynes, C. Steele, M. Botto, S. Gordon, and G.D. Brown. 2007. Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat Immunol* 8:31-38.

Tomalka, J., S. Ganesan, E. Azodi, K. Patel, P. Majmudar, B.A. Hall, K.A. Fitzgerald, and A.G. Hise. 2011. A novel role for the NLRC4 inflammasome in mucosal defenses against the fungal pathogen Candida albicans. *PLoS Pathogens* 7:e1002379.

Turchinovich, G., and D.J. Pennington. 2011. T cell receptor signalling in gammadelta cell development: strength isn't everything. *Trends Immunol* 32:567-573.

Tyznik, A.J., J.C. Sun, and M.J. Bevan. 2004. The CD8 population in CD4-deficient mice is heavily contaminated with MHC class II-restricted T cells. *The Journal of Experimental Medicine* 199:559-565.

Urban, C.F., U. Reichard, V. Brinkmann, and A. Zychlinsky. 2006. Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. *Cell Microbiol* 8:668-676.

van de Veerdonk, F.L., L.A. Joosten, P.J. Shaw, S.P. Smeekens, R.K. Malireddi, J.W. van der Meer, B.J. Kullberg, M.G. Netea, and T.D. Kanneganti. 2011. The inflammasome drives protective Th1 and Th17 cellular responses in disseminated candidiasis. *Eur J Immunol* 41:2260-2268.

van de Veerdonk, F.L., R.J. Marijnissen, B.J. Kullberg, H.J. Koenen, S.C. Cheng, I. Joosten, W.B. van den Berg, D.L. Williams, J.W. van der Meer, L.A. Joosten, and M.G. Netea. 2009. The macrophage mannose receptor induces IL-17 in response to Candida albicans. *Cell Host Microbe* 5:329-340.

van de Veerdonk, F.L., M.G. Netea, T.J. Jansen, L. Jacobs, I. Verschueren, J.W. van der Meer, and B.J. Kullberg. 2008. Redundant role of TLR9 for anti-Candida host defense. *Immunobiology* 213:613-620.

van der Graaf, C.A., M.G. Netea, B. Franke, S.E. Girardin, J.W. van der Meer, and B.J. Kullberg. 2006. Nucleotide oligomerization domain 2 (Nod2) is not involved in the pattern recognition of Candida albicans. *Clin Vaccine Immunol* 13:423-425.

Vautier, S., G. Sousa Mda, and G.D. Brown. 2010. C-type lectins, fungi and Th17 responses. *Cytokine Growth Factor Rev* 21:405-412.

Veerdonk, F.L.v.d. 2011. STAT1 Mutations in Autosomal Dominant Chronic Mucocutaneous Candidiasis. *New England Journal of Medicine* 365:

Villamon, E., D. Gozalbo, P. Roig, C. Murciano, J.E. O'Connor, D. Fradelizi, and M.L. Gil. 2004. Myeloid differentiation factor 88 (MyD88) is required for murine resistance to Candida albicans and is critically involved in Candida -induced production of cytokines. *Eur Cytokine Netw* 15:263-271.

von Bernuth, H., C. Picard, Z. Jin, R. Pankla, H. Xiao, C.L. Ku, M. Chrabieh, I.B. Mustapha, P. Ghandil, Y. Camcioglu, J. Vasconcelos, N. Sirvent, M. Guedes, A.B. Vitor, M.J. Herrero-Mata, J.I. Arostegui, C. Rodrigo, L. Alsina, E. Ruiz-Ortiz,

M. Juan, C. Fortuny, J. Yague, J. Anton, M. Pascal, H.H. Chang, L. Janniere, Y. Rose, B.Z. Garty, H. Chapel, A. Issekutz, L. Marodi, C. Rodriguez-Gallego, J. Banchereau, L. Abel, X. Li, D. Chaussabel, A. Puel, and J.L. Casanova. 2008. Pyogenic bacterial infections in humans with MyD88 deficiency. *Science* 321:691-696.

Wells, C.A., J.A. Salvage-Jones, X. Li, K. Hitchens, S. Butcher, R.Z. Murray, A.G. Beckhouse, Y.L. Lo, S. Manzanero, C. Cobbold, K. Schroder, B. Ma, S. Orr, L. Stewart, D. Lebus, P. Sobieszczuk, D.A. Hume, J. Stow, H. Blanchard, and R.B. Ashman. 2008. The macrophage-inducible C-type lectin, mincle, is an essential component of the innate immune response to Candida albicans. *J Immunol* 180:7404-7413.

Werner, J.L., M.A. Gessner, L.M. Lilly, M.P. Nelson, A.E. Metz, D. Horn, C.W. Dunaway, J. Deshane, D.D. Chaplin, C.T. Weaver, G.D. Brown, and C. Steele. 2011. Neutrophils produce interleukin 17A (IL-17A) in a dectin-1- and IL-23-dependent manner during invasive fungal infection. *Infection and Immunity* 79:3966-3977.

Werninghaus, K., A. Babiak, O. Gross, C. Holscher, H. Dietrich, E.M. Agger, J. Mages, A. Mocsai, H. Schoenen, K. Finger, F. Nimmerjahn, G.D. Brown, C. Kirschning, A. Heit, P. Andersen, H. Wagner, J. Ruland, and R. Lang. 2009. Adjuvanticity of a synthetic cord factor analogue for subunit Mycobacterium tuberculosis vaccination requires FcRgamma-Syk-Card9-dependent innate immune activation. *The Journal of Experimental Medicine* 206:89-97.

Willment, J.A., and G.D. Brown. 2008. C-type lectin receptors in antifungal immunity. *Trends Microbiol* 16:27-32.

Wisplinghoff, H., T. Bischoff, S.M. Tallent, H. Seifert, R.P. Wenzel, and M.B. Edmond. 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin Infect Dis* 39:309-317.

Wright, S.W., and R.A. Johnson. 1997. Human immunodeficiency virus in women: mucocutaneous manifestations. *Clin Dermatol* 15:93-111.

Yang, X.O., A.D. Panopoulos, R. Nurieva, S.H. Chang, D. Wang, S.S. Watowich, and C. Dong. 2007. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J Biol Chem* 282:9358-9363.

Yang, X.P., K. Ghoreschi, S.M. Steward-Tharp, J. Rodriguez-Canales, J. Zhu, J.R. Grainger, K. Hirahara, H.W. Sun, L. Wei, G. Vahedi, Y. Kanno, J.J. O'Shea, and A. Laurence. 2011. Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. *Nat Immunol* 12:247-254.

Ye, P., F.H. Rodriguez, S. Kanaly, K.L. Stocking, J. Schurr, P. Schwarzenberger, P. Oliver, W. Huang, P. Zhang, J. Zhang, J.E. Shellito, G.J. Bagby, S. Nelson, K.

Charrier, J.J. Peschon, and J.K. Kolls. 2001. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J Exp Med* 194:519-527.

Yeh, N., N.L. Glosson, N. Wang, L. Guindon, C. McKinley, H. Hamada, Q. Li, R.W. Dutton, P. Shrikant, B. Zhou, R.R. Brutkiewicz, J.S. Blum, and M.H. Kaplan. 2010. Tc17 cells are capable of mediating immunity to vaccinia virus by acquisition of a cytotoxic phenotype. *J Immunol* 185:2089-2098.

Yuan, X., and K.R. Wilhelmus. 2010. Toll-like receptors involved in the pathogenesis of experimental Candida albicans keratitis. *Invest Ophthalmol Vis Sci* 51:2094-2100.

Zelante, T., A. De Luca, P. Bonifazi, C. Montagnoli, S. Bozza, S. Moretti, M.L. Belladonna, C. Vacca, C. Conte, P. Mosci, F. Bistoni, P. Puccetti, R.A. Kastelein, M. Kopf, and L. Romani. 2007. IL-23 and the Th17 pathway promote inflammation and impair antifungal immune resistance. *Eur J Immunol* 37:2695-2706.

Zelante, T., R.G. Iannitti, A. De Luca, J. Arroyo, N. Blanco, G. Servillo, D. Sanglard, U. Reichard, G.E. Palmer, J.P. Latge, P. Puccetti, and L. Romani. 2012. Sensing of mammalian IL-17A regulates fungal adaptation and virulence. *Nat Commun* 3:683.

Zenaro, E., M. Donini, and S. Dusi. 2009. Induction of Th1/Th17 immune response by Mycobacterium tuberculosis: role of dectin-1, Mannose Receptor, and DC-SIGN. *J Leukoc Biol* 86:1393-1401.

Zielinski, C.E., F. Mele, D. Aschenbrenner, D. Jarrossay, F. Ronchi, M. Gattorno, S. Monticelli, A. Lanzavecchia, and F. Sallusto. 2012. Pathogen-induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta. *Nature* 484:514-518.

Zou, Q., X. Yao, J. Feng, Z. Yin, R. Flavell, Y. Hu, G. Zheng, J. Jin, Y. Kang, B. Wu, X. Liang, C. Feng, H. Liu, W. Li, X. Wang, Y. Wen, and B. Wang. 2011. Praziquantel facilitates IFN-gamma-producing CD8+ T cells (Tc1) and IL-17-producing CD8+ T cells (Tc17) responses to DNA vaccination in mice. *PLoS One* 6:e25525.