A Thesis

entitled

Role of Serum Amyloid A3 Proteins in Antifungal Immune Responses during Oropharyngeal Candidiasis

by

Priosmita Biswas

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Master of Science Degree in Biology

_____

Dr. Heather Conti, Committee Chair Dr.

_____

Deborah Vestal, Committee Member

_____

Dr. Song Tao-Liu, Committee Member

_____

Dr. Deborah Chadee, Committee Member

_____

Dr. John Plenefisch, Acting Chair,
Department of Biological Sciences

_____

Dr. Amy Thompson, Acting Dean
College of Graduate Studies

The University of Toledo

December 2021

An Abstract of

Role of Serum Amyloid A3 Proteins in Antifungal Immune Responses during Oropharyngeal Candidiasis

by

Priosmita Biswas

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Master of Science Degree in
Biology

The University of Toledo
December 2021

Candida albicans (*C. albicans*) is a commensal fungal pathogen of human skin and mucosa that causes oropharyngeal candidiasis (OPC), a severe fungal infection of the oral cavity in immunocompromised patients (Conti & Gaffen, 2015). As a result, clinical understanding of the host immune responses against *C. albicans* is critical. IL-17 is an important pro-inflammatory cytokine known to have a protective role against oral candidiasis by modulating chemokines, antimicrobial peptides, and downstream acute-phase proteins required for fungal clearance (Mengesha & Conti, 2017). The present study identified serum amyloid A3 (SAA3) proteins which are a family of acute phase apolipoproteins as being highly induced in wild-type infected mice with *C. albicans.* Moreover, we show *Saa3*$^{-/-}$ (KO) mice are more susceptible to oral candidiasis with high fungal burden than *Saa3*$^{+/+}$ (WT) mice suggesting the involvement of SAA3 in protection against OPC. However, *Saa3*$^{-/-}$ (KO) mice at later time points were observed to clear the fungal load. Additionally, the expression of other serum amyloid genes (*Saa1* & *Saa2*) known to have an antibacterial effect are found not to be compensating for the lack of Saa3 in *Saa3*$^{-/-}$ (KO) mice in clearing fungal infection. This led us to hypothesize that genes

downstream of IL-17RA signaling pathway may be involved in antifungal immunity. At early time points following OPC infection, the expression level of *Il1β* and *Il-6* was reduced followed by *Il-17a*, indicating that *Saa3*$^{-/-}$ (KO) mice have impairments towards antifungal immunity by having high fungal burden. However, the expression of all these genes along with *Defb3* was found to be elevated in *Saa3*$^{-/-}$ (KO) mice at later time points. Besides having susceptibility to OPC in *Saa3*$^{-/-}$ (KO) mice there were also defects in immune cell population including neutrophils observed, compared to *Saa3*$^{+/+}$ (WT) OPC mice. Thus, the findings demonstrated that SAA3 plays a role during OPC, but the clearance of fungal infection in *Saa3*$^{-/-}$ (KO) mice is dependent on an increase in expression of antifungal genes at later time points known to be involved in anti-Candida immunity.

Acknowledgments

First and foremost, I want to express my gratitude to Dr. Heather Conti, my mentor, for her advice, support, and encouragement during my time as a graduate student. I would also like to express my gratitude to my committee members – Dr. Deborah Vestal, Dr. Song Tao-Liu, and Dr. Deborah Chadee for being a part of my thesis committee and their valuable insight and suggestions.

I want to express my gratitude to all the members of the Conti lab with whom I have worked and interacted over the years. From my current lab members Dylan Launder and John Dillon to my former lab members Dr. Jess Saul-McBeth, Katherine Mae McKeone, and Nathan Schmidt. I would also thank many undergraduate researchers with whom I worked with.

Finally, I would like to thank my family members and my in-laws, my husband for their constant love and support throughout my entire journey here in the United States. I cannot even express how much my spouse has done and continues to do for me. Thank you everyone for your unwavering assistance and motivation.

From hard night studies to long drives here in Toledo exploring the city, I want to thank all of my friends too. Thank you to everyone who contributed to making my experience so memorable and colorful.

Table of Contents

List of Figures

List of Abbreviations

AMP.....Antimicrobial peptide
ANOVA .....Analysis of Variance
APP .....Acute-phase proteins
APR.....Acute-phase response

C. albicans .....*Candida. Albicans*
CD4+.....Candida Pseudo hyphal regulator 1
cDNA .....Complementary DNA
CFU.....Cluster of Differentiation 4
CLR.....Colony Forming Unit
CPH1.....C-Type Lectin Receptor

Defb3.....Murine *β-Defensin 3* Gene
DNA.....Deoxyribonucleic Acid

EFG1 .....Enhanced Filamentous Growth 1

GAPDH.....Glyceraldehyde 3-phosphate dehydrogenase

H&E .....Hematoxylin and Eosin
HIV .....Human Immunodeficiency Virus

IL-17 .....Interleukin-17
IL-17A Interleukin-17
IL-17A/F .....Interleukin-17A and Interleukin-17F Heterodimeric Complex
IL-17RA/RC .....Interleukin-17 Receptor A and Interleukin-17 Receptor C Heterodimeric Receptor Complex
IL-6 .....Interleukin-6
IL-1β Interleukin-1β
IL-1a.....Interleukin-1alpha

KO.....Knock Out (-/-)

NF-kb .....Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NLRs .....NOD-like receptors
nTh .....Natural T Helper

OD.....Optical Density
OEC.....Oral Epithelial Cells
OPC.....Oropharyngeal Candidiasis

PAMP.....Pathogen Associated Molecular Patterns
PBS .....Phosphate Buffered Saline
PRR.....Pattern Recognition Receptor
qPCR.....Quantitative Real-Time PCR

RLRs .....RIG-I-like receptors

SAA.....Serum Amyloid A
SAA1.....Serum Amyloid A1 Protein-coding gene
SAA2.....Serum Amyloid A2 Protein-coding gene
SAA3.....Serum Amyloid A3 pseudogene
Saa3$^{-/-}$.....Represents KO mice (absence of mouse *Saa3* gene)
Saa3$^{+/+}$.....Represents WT mice (presence of mouse *Saa3* gene)

TGF.....Transforming Growth Factor
Th .....T Helper
TLR.....Toll-like Receptor
TNF.....Tumor Necrosis Factor

WT .....Wild Type (+/+)

YPD.....Yeast Peptone Dextrose

List of Symbols

°C .....Degrees Celsius
ml .....Milliliter
n.....Total number of mice
ng.....Nano gram
α .....Alpha
β .....Beta
δ.....Delta
µL.....Microliter

Chapter 1

Literature Review

1.1 An Introduction to *Candida albicans* and Various Forms of Candidiasis caused by *Candida albicans*

Candida albicans (C. albicans) is a commensal fungal pathogen that resides mostly in healthy individuals. It colonizes the gastrointestinal, genital mucosa as well as human skin as commensal in roughly 80% of the general population (Vila et al., 2020). However, when the balanced homeostasis of the fungal commensal *Candida* is disrupted, it causes an overgrowth of *Candida albicans* to cause candidiasis (Singh et al., 2015). The different forms of candidiasis include oropharyngeal (oral cavity), disseminated, vulvovaginal (vagina), and cutaneous candidiasis (skin) (Mayer et al., 2013). However, *Candida* infection can be fatal if it reaches the circulation and becomes systemic (Pfaller & Diekema, 2007). This bloodstream infection (BSI) is the fourth most common cause of infections in hospitals around the United States (Pfaller & Diekema, 2007). Moreover, oropharyngeal candidiasis (OPC) (also known as oral thrush) is a known superficial fungal infection caused by *C. albicans* commonly found in immunocompromised individuals with HIV/AIDS, diabetes, and those who have received head-neck irradiation (Conti & Gaffen, 2015) (Pankhurst, 2013) (Pfaller & Diekema, 2007).

Although *Candida albicans* is still the most frequently recovered yeast from the oral cavity, OPC can also be caused by a variety of non-*C. albicans* species such as *C.*

glabrata, C. tropicalis, C. krusei, C. parapsilosis, and C. dubliniensis. These species are often recovered from a mixed population usually in combination with *C. albicans* (Sullivan & Coleman, 1998) (Cannon & Chaffin, 1999).

Both the innate and adaptive immune systems are involved in protection against fungal infections. The high incidence of OPC in HIV/AIDS patients is further associated with a defect in CD4+T lymphocytes, making it important in the host immune response against *Candida* infection in the oral cavity (Rabeneck et al., 1993). Therefore, Th17 cells, a subset of CD4+ T cells, are found to be critical for antifungal immunity (Conti et al., 2009). Further research led to the identification of the pro-inflammatory cytokine interleukin-17 (IL-17A) which plays a key role in the clearance of fungal infections from the oral cavity (Conti et al., 2016). Importantly, many hematopoietic and non-hematopoietic cells, have a role in immunological fitness throughout the IL-17 signaling cascade including CD4+ T helper 17 cells (Th17), natural Th17 cells, γδ-T cells, neutrophils, dendritic cells (DCs), and oral epithelial cells (OECs) (Conti et al., 2016).

However, on the other hand, later the contribution of innate immunity, was found to have a bigger role in fungal infection on mucosal surfaces. In this regard, IL-17 mediated antifungal innate immunity with natural Th17 cells and γδ-T cells play a critical role in protection against OPC (Mengesha & Conti, 2017). Therefore, besides many effector molecules downstream of the antifungal signaling pathway, also includes other downstream proteins such as antimicrobial peptides (AMPs) and acute phase proteins (APPs) whose roles in antifungal immunity are further discussed.

1.2 Structure & mechanism of *Candida albicans* towards pathogenicity

Candida albicans is a polymorphic fungus that can alternate between ovoid yeast, elongated ellipsoid cells (pseudo hyphae), or filamentous form true hyphae (Boyce & Andrianopoulos, 2015). The cell wall of *C. albicans* is composed of carbohydrates and proteins (Gow et al., 2011). The glucans with chitin are commonly found inside an outer mannan layer that makes up the structure of the fungal cell, which is a dynamic structure. The glycosylated mannoproteins secreted from the cell surface from the mannan layer, aids in cell-cell recognition (Hall, 2015). These mannoproteins are covalently linked to the β 1-3 glucans directly, or through β 1-6 glucans (Cannon & Chaffin, 1999). The cell wall plays an important role with cell wall proteins (CWPs) to be critical during pathogenesis. Adhesins are chemicals that aid in the attachment of fungal cells to their host cells (Calderone & Fonzi, 2001) which includes the Als family, and Hwp1 as the most important (Tsuchimori et al., 2000). There are also several hydrolytic secretory proteins, such as Saps and phospholipase, that play a crucial role in host tissue invasion (Naglik et al., 2003). The four Agglutinin-like sequences (Als) proteins include (Als1, Als2, Als3, and Als4) that act as adhesins under different conditions with overlapping functions (Hoyer et al., 2008). On the other hand, hyphal cell wall protein (Hwp1), is only expressed in the hyphal cells (Staab et al., 2013). Apart from Als family, the Secreted Aspartyl Protease (Sap) family of proteins includes ten members (Sap 1- Sap 10) and is considered as the second-largest family of secretory proteins that have been thoroughly studied (De Bernardis et al., 1999). Saps are

hydrolytic enzymes that cause epithelial cell invasion, which leads to tissue injury (De Bernardis et al., 1999).

To cause disease, *C. albicans* need to adhere to specific surfaces, resist the host's innate immune responses followed by proliferation and spreading into the host body. The ability to switch between yeast and hyphal forms (dimorphism) is critical for pathogenesis, allowing *C. albicans* to invade and elude immune responses in a range of body sites (Mayer et al., 2013). The transcription factors involved in switching between yeast and hyphal forms are Enhanced Filamentous Growth 1 (EFG1) and Candida Pseudo hyphal regulator 1 (CPH1) (Cheng et al., 2012). EFG1 acts as a key regulator of hyphal morphogenesis (Martin et al., 2011). The adherence of *C. albicans* to the host surface is critical, followed by an invasion of the host's tissue. The steps involved in tissue invasion by *C. albicans* include adhesion to the epithelium, epithelial penetration, and vascular dissemination, which entails hyphal penetration and seeding of yeast cells into the bloodstream that finally leads to dissemination (Gow et al., 2011)

1.3 Pattern Recognition Receptors of *Candida albicans*

Pathogens are recognized by host cell surface receptors, which activate intracellular signaling pathways, resulting in the activation of inflammatory mediators. These are known as pattern recognition receptors (PRRs) which recognize a range of pathogen-associated molecular patterns (PAMPs) generated by invading microbes to identify pathogens and initiate the immune response. PAMP–PRR interactions on myeloid cells further activate downstream pathways that kill the pathogen (Akira et al., 2006). PAMPs present on *C. albicans* cell walls mostly contain O- and N-linked glycoproteins, as well as the skeletal

polysaccharides chitin, β-1,3-glucan, and β-1,6-glucan. There are four families of PRRs, which include the Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs) (Netea et al., 2006). TLRs are expressed by various immune and non-immune cells including B-cells, T-cells, dendritic cells, epithelial cells, and fibroblasts (Akira et al., 2006). Among them, TLR2, TLR4, and TLR6 identify mannoprotein components on the cell wall of *C. albicans* (Netea et al., 2015). Further, TLR2 forms a heterodimer with TLR1 or TLR6 while the majority of TLRs form homodimers (Patin et al., 2019). TLR2 and TL4 are primarily found on myeloid cells including dendritic cells, monocytes, and macrophages, although TLR4 has also been discovered on B cells, mast cells, and neutrophils (Vaure & Liu, 2014). Dectin 1 is the most well-studied glucan receptor which detects glucan present in the *Candida* cell wall, while Dectin-2 detects mannan (Hardison & Brown, 2012)**.**

During infections, oral epithelial cells (OECs) serve as the first physical barrier (Cheng et al., 2012) where TLR2 and TLR4 are mostly expressed on their surface (Naglik & Moyes, 2011) (Weindl et al., 2011). *C.albicans* is initially identified by TLRs on OECs, triggering a signaling cascade. This cascade leads to the production of more cytokines downstream, as well as the recruitment of chemokines (CXCL1, CXCL2, CXCL5) to recruit neutrophils and antimicrobial peptides (AMPs) to directly kill Candida, which leads to fungal control (Ge et al., 2020) (Cheng et al., 2012).

1.4 Role of IL-17 in Antifungal immunity

Antifungal immunity against OPC caused by *C. albicans* in both humans and mice is mainly mediated by the proinflammatory cytokine interleukin-17 (IL-17). T helper cells

act as an important source of IL-17 during fungal infection (Korn et al., 2009). Differentiation of Th17 cells from naïve CD4+ T-cells is mediated by signaling cytokines such as transforming growth factor-beta (TGF-β), IL-6, and IL-1β in presence of IL-23 to maintain and expand Th17 cells (Hernandez-Santos & Gaffen, 2012). Because of the relevance of IL-17 for *Candida* clearance and the time it takes for IL-17-producing Th17 cells to differentiate, it was unclear whether Th17 might be a substantial source of IL-17 for the innate response or not, during OPC. Therefore, studying the early sources of IL-17 implicated in *Candida* clearance before CD4+ cells convert into Th17 cells producing IL-17 (Cua & Tato, 2010) has been a focus of study.

Moreover, in an acute model of oral candidiasis, the early response of γδ T cells and nTh17 against oral *Candida* infections was further found to be protective (Conti & Gaffen, 2015) which have also been schematically represented (Fig 1.4). More significantly, gamma-delta (γδ) T cells have been identified as a substantial innate source of IL-17 before the adaptive Th17 cell response is activated after fungal infection (Martin-Orozco et al., 2009).

The effects of IL-17 signaling is initiated by binding to its receptors that further trigger antifungal immunity, showing the importance of the IL-17 receptor during oral candidiasis. The family consists of six cytokines (IL-17A, IL-17B, IL-17C, IL-17D, IL-E, and IL-17F) and five receptors (IL-17RA, IL-17RB, IL-17RC, IL-17RD, and IL-17RE) (Monin & Gaffen, 2018). The main cytokine, IL-17, has pleiotropic effects. IL-17A and IL-17F are the most closely related as they bind to the same receptor (IL-17RA and IL-RC) and play a role in fungal clearance during OPC (Conti & Gaffen, 2015) (Toy et al., 2006). The IL-17RA receptor is one of the common components of numerous additional

IL-17 receptor complexes that bind various IL-17 cytokine family members and is extensively expressed on hematopoietic and nonhematopoietic cells, including endothelial and epithelial cells (Gaffen, 2009). IL-17A and IL-17F signaling further promote the expression of proinflammatory cytokines (TNFα, IL-1β, G-CSF, GM-CSF), antimicrobial peptides (S1008, β-defensin 3), and chemokines for neutrophil recruitment (CXCL 1, 2, and 5) required for fungal clearance in the host. Further downstream signaling cascades are triggered by IL-17 signaling via its receptors IL-17RA and IL-17RC by binding to SEFIR-containing ACT1 adaptor to mediate a range of downstream processes such as the MAPK, CEPBβ, and NF-κB pathways. β-defensin 3 which is antimicrobial peptides that kills invading pathogens directly (Mengesha & Conti, 2017) (Conti et al., 2009) (Conti et al., 2016) (Bonass et al., 1999).

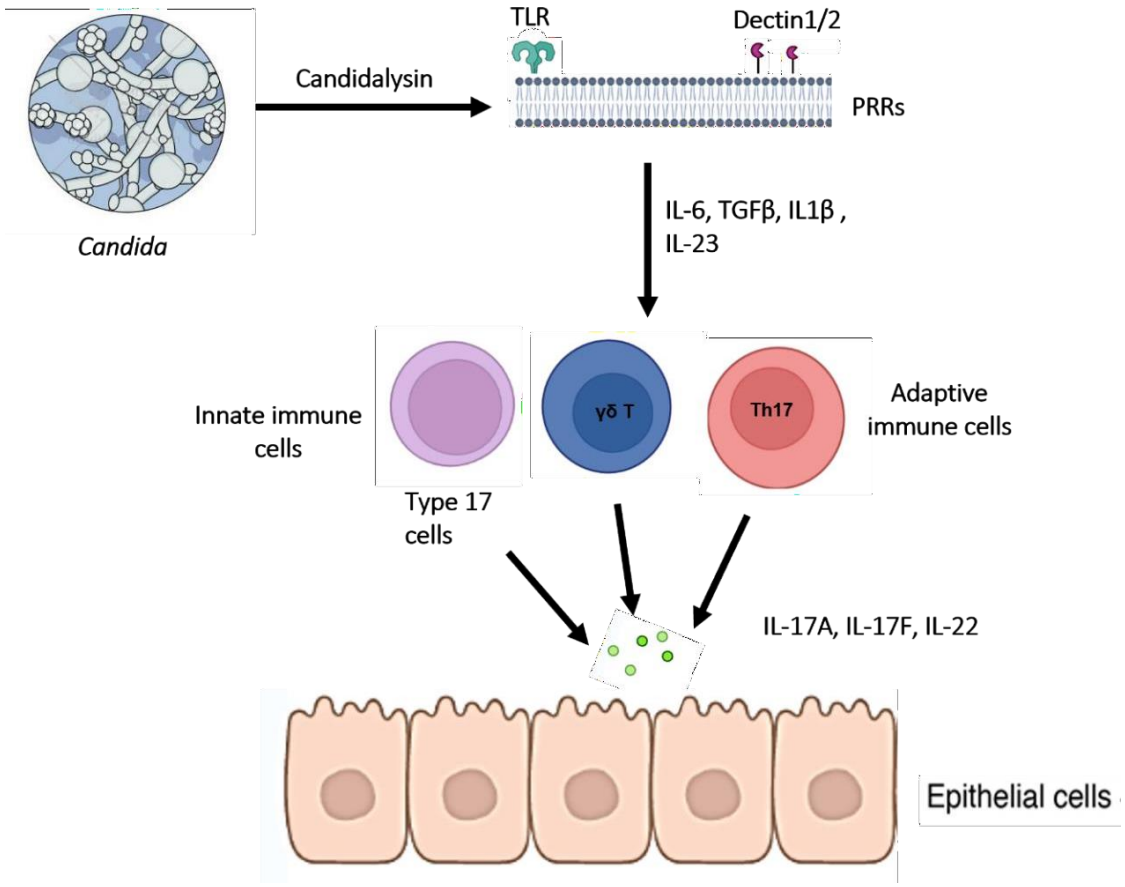


Figure 1.4: Schematic representation showing IL-17 signaling pathway involved in antifungal immunity. *C.albicans* are sensed by PRRs, such as Dectin 1 & Dectin 2 found on epithelial cells. Exposure of *C.albicans* triggers the release of signaling cytokines (IL-6, TGF β, IL-1β, IL-23) leading to CD4+ differentiation into IL-17 producing cells such as innate nTh17, γδ T, and adaptive Th17 (Conti & Gaffen, 2015).

When compared to immunocompetent mice, animals missing the IL-17RA (*IL17ra*$^{-/-}$) component are unable to develop an immune response and clear infection (Conti et al., 2009)**.** This highlights the significance of IL-17 signaling via this receptor in the oral cavity in the host's antifungal defense, which explains why *IL17ra*$^{-/-}$ mutant animals are more vulnerable to OPC.

Besides modulating chemokines, which lead to neutrophil recruitment, IL-17 also controls the synthesis of many proteins such as antimicrobial peptides (AMPs) (Mengesha

& Conti, 2017) and acute phase proteins (APPs). Additionally, previous analysis confirmed that during OPC infection there is a high induction of acute-phase proteins observed in WT *C.albicans* infected mice (Conti et al., 2009). Therefore, studying the importance of acute-phase proteins during OPC towards antifungal immunity has been a point of research interest.

1.5 Acute Phase Response (APR) and Acute Phase Proteins (APP)

The acute phase response (APR) is an important part of the innate immune response which is often caused by infection, inflammation, trauma, tissue damage, or the introduction of immunogens such as bacterial lipopolysaccharide (LPS) (O'Reilly & Eckersall, 2014). APR is often triggered by pro-inflammatory signals (cytokines and chemokines) produced by the activation of macrophages, monocytes, endothelial cells, and T-cells (Cray et al., 2009).

Acute-phase proteins (APPs) are known to be the hallmark of the acute-phase response (APR) released from the liver (Cray et al., 2009). These (APPs) proteins are also known to be expressed from a wide range of extrahepatic tissues other than the liver which enhances the APR like adipocytes & macrophages (Schrodl et al., 2016). The extrahepatic release of APP has not been widely studied in humans (Jain et al., 2011) however it is thought that the APP plays a key role in local inflammatory reactions (Schrodl et al., 2016). Two types of APPs occur with APR variations in serum concentration. Hepatocytes classify a group of individuals with high production as "positive" APP and a group with low production as "negative" APP. The APP pattern differs from species to species, and its

amount of expression is species-specific (Cray et al., 2009). The positive APP is further subdivided into different categories depending on the level of increase (Jain et al., 2011). Low APP levels can increase up to 10-fold, while significant APP levels which can increase more than 100-fold, generally within the first 48 hours after the triggering event, like C reactive protein (CRP) and serum amyloid A (SAA) (Sproston & Ashworth, 2018). Another component of APP is negative APP, which is comprised of albumin and transferrin. The lower APR expression is related to the enhanced capacity of the liver to synthesize positive APP, which directly participates in the immune response (Steel & Whitehead, 1994).

However, there is always a chance of having detrimental effects due to the prolonged inflammatory response caused by the APPs. Another element of the APR is the production of glucocorticoids, which are frequently recognized to mediate high concentrations of APP which increases adrenocorticotrophic hormone secretion (Jain et al., 2011). The importance of corticosterone in humans includes modulating proteins that are synthesized in the liver as a part of the APR. As a result, based on a study, an increase in corticosterone is noticed later than the arrival of IL-6 (Jain et al., 2011). It has also been known that the administration of corticosterone can induce APP synthesis even in the absence of an inflammatory response (Curtis & Butler, 1980), which shows that glucocorticoids may have an effect independent of cytokines. Thus, glucocorticoids, which play a crucial role in the initiation of APR, further triggers negative feedback as the process progresses.

1.6 Acute Phase Proteins: Serum Amyloid A (SAA)

SAA proteins were discovered in the early 1970s when antibodies were developed against a peptide produced during amyloidosis-related inflammation (AA amyloid) that interacted with a protein present in acute phase plasma (Kuret et al., 2018). The deposition of fibrils in the extracellular space and organs characterizes AA amyloidosis, which typically complicates chronic inflammatory diseases mostly associated with the persistent acute phase response (Lachmann et al., 2007).

Serum amyloid A is a family of acute phase apolipoproteins produced by hepatocytes and extrahepatic tissues, which are released into the circulation during trauma, infection, and stress. They are thought to have antibacterial activities by having a protective role during many bacterial and viral infections (Zheng et al., 2020). Moreover, the SAA family is further classified into two types: acute phase SAAs and constitutive SAAs (Jain et al., 2011). During inflammation, infection, or trauma the levels of acute-phase SAAs are highly increased (Eckersall et al., 2001). Normal human serum A-SAA concentrations are less than 1 mg/L, however, during the acute phase response, they are drastically increased up to 1000 mg/L (1000-fold) (Couderc et al., 2017). On the other hand, during acute phase response, the levels of constitutive SAA do not change (Uhlar & Whitehead, 1999). The SAA family member has been found in a variety of species; however, it has primarily been investigated in humans and mice because of high sequence homology (Santiago-Cardona et al., 2003). The human genome is composed of four members, two A-SAAs (SAA1 and SAA2), one pseudogene (SAA3), and one constitutive form (SAA4) (Ather et al., 2018). Similarly, in mice, four members are found, and in that case, SAA3 is a normal and functional protein. However other species, including the dog, rabbit, cow, sheep, and goat,

have been described as having more than one member of the family (Fan et al., 2020) (Uhlar & Whitehead, 1999).

In general, the SAA protein sequence is highly conserved in all the family isoforms and among different species comprises 104 and 112 amino acid residues with 18 amino acid signal peptides (Uhlar & Whitehead, 1999). However, in humans, SAA1 and SAA2 proteins are the most similar forms with approximately 90% of homology. The constitutive form (SAA4) contains additional 8 amino acid insertions that are not found in the acute phase isoforms and shares nearly 53-55% of the amino acids with SAA1 and SAA2 (Uhlar & Whitehead, 1999). Human SAA3 is a pseudogene, although mouse SAA3 is totally functional which shares 69% homology with human SAA1 and SAA2 (Tannock et al., 2018) makes SAA3 an excellent model to study human-related diseases. The genes are normally found in clusters on chromosome 11 in humans and chromosome 7 in mice (Uhlar & Whitehead, 1999).

Several immune-related functions have been documented for the SAA family, but among them bacterial opsonization is well-documented. SAA has been known to have antimicrobial properties, mostly to be found by binding to the outer surface of Gram-negative bacteria such as *Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Vibrio cholerae* but not Gram-positive bacteria (Hari-Dass et al., 2005). They appear to contain an outer membrane protein A (OmpA) responsible for the binding of SAA, which would function as an opsonin for enhanced invading bacterial absorption by neutrophils (Shah et al., 2006). Additionally, binding to SAA-opsonized bacteria further lead to the production of cytokines such as TNF- α and IL-10 which suggest a significant role of SAA proteins in the host defense against invading

bacteria (Shah et al., 2006). Similarly, in past studies during fungal infection, induction of many SAA proteins has been documented (Conti et al., 2009) however the mechanism towards antifungal immunity is poorly understood.

1.7 Acute Phase Proteins: Serum Amyloid A3 (SAA3)

Serum amyloid A (SAA) are small proteins exerting a wide range of functions. Besides playing as a biomarker of inflammation, SAAs act as critical mediators of disease pathogenesis (Jain et al., 2011). The levels of the SAA proteins increase during injury, and if the injury does not continue, the levels return to normal after 7-10 days (Jain et al., 2011). Because SAA is known to be produced extrahepatically, it may be involved in a condition that does not result in systemic acute phase response, but rather plays an effective role at the site of expression (Uhlar & Whitehead, 1999). Moreover, the antibacterial activity of SAA proteins in Gram-negative bacteria is well documented in previous studies (Hari-Dass et al., 2005).

SAA1 and SAA2 are predominately synthesized in the liver and are released into circulation (Jain et al., 2011). In the mice model, SAA1 has only been documented in the liver and kidney and SAA2 in the intestine (Benditt & Meek, 1989) but SAA3 is produced in a wide range of tissues (Fan et al., 2020). In multiple studies, in mice have shown that SAA3 has a role in bacterial infections such as *Pseudomonas* (Fan et al., 2020). However, in rats, SAA3 mRNA, not SAA1/2 mRNA, was identified extrahepatically following LPS exposure in the lung, ileum, and intestine (Benditt & Meek, 1989) (Marhaug et al., 1997). Many investigations have indicated that SAA has cytokine-like properties that are involved in a variety of inflammatory, immunologic, and protective pathways (Jain et al., 2011). In

humans, A-SAA expression is limited to SAA1 and SAA2, with the SAA3 isoform formerly thought to be non-expressed due to the lack of mRNA (Uhlar & Whitehead, 1999). More recently, it has been discovered that the induction and appearance in plasma of all three acute-phase SAA isoforms (SAA1, SAA2, and SAA3) share a high degree sequence homology and function, but the way of induction and appearance in plasma are different (Chait et al., 2020).

In mice, SAA3 is known to have antibacterial properties (Fan et al., 2020). Even though mouse SAA3 is abundant in inflammatory tissues, it is found not to have any amyloidogenic properties nor does it contribute to the rise in plasma SAA levels during APR (Chiba et al., 2009). However, later research found SAA3 mRNA expression in mammalian epithelial cell lines, in response to prolactin and LPS (Larson et al., 2003). Additionally, the amino acid sequences of mouse SAA3 exhibited a high degree of similarity to the human isoform SAA1 (69% amino acid identity) which suggests using this as an excellent model in studying human diseases (Tannock et al., 2018).

Additionally, during OPC, using microarray analysis, when compared to *IL17ra-/-* OPC mice it has been found that a whole host of genes along with acute-phase proteins were upregulated in WT *Candida albicans* infected mice (Conti et al., 2009). This was further supported by the induction of *Saa3* gene expression in WT *Candida albicans* infected mice by qPCR analysis (Fig 1.7A). Thus, this induction of *Saa3* during OPC motivated us to study its function during fungal pathogenesis which has been point of a research interest so as to understand the potential role towards antifungal immunity.

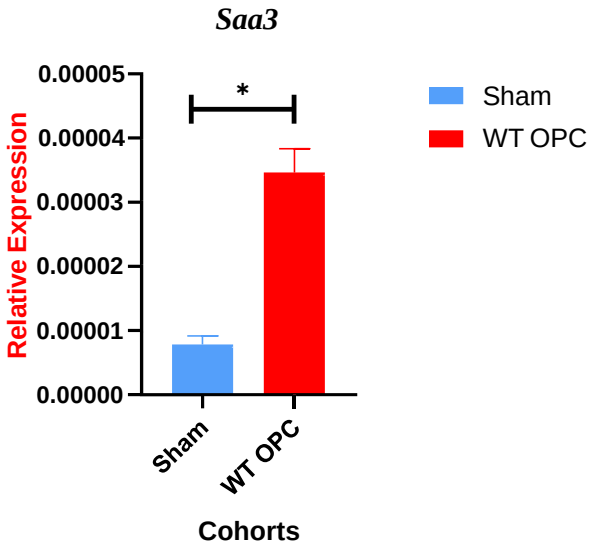


Figure 1.7: *Saa3* expression in *C. albicans* infected *Saa3*$^{+/+}$ (WT) mice compared to sham mice. RNA extracted from tongue tissue harvested from *C. albicans* infected WT mice and control (Sham) was used for gene expression studies. Data showed is a representative of three total experiments. Statistical analysis was performed using ANOVA, *P<0.05 (qPCR n=3-5) where (n= total number of mice in each group used in this experiment).

1.8 Hypothesis & Objectives

Oropharyngeal candidiasis (OPC) is a known superficial fungal infection mainly found on mucosal surfaces caused by *C. albicans* common in immunocompromised patients such as HIV/AIDS, immunosuppressed patients under prolonged exposure to antibiotics & corticosteroids, and cancer patients undergoing chemotherapy and radiotherapy. These patients pose significant challenges as they have defects in the IL-17RA signaling pathway causing them more susceptible to OPC. (Conti & Gaffen, 2015). During OPC, there are many proinflammatory cytokines and chemokines that are known to have a protective role (Conti & Gaffen, 2015). However, besides these proinflammatory signaling molecules, there are many proteins (acute-phase proteins) that were observed to be highly induced in *Candida albicans* infected mice which motivated us to move towards SAA3, to understand its role during fungal infection.

Therefore, this project was based on two specific aims where the first aim was to study the protective role of SAA3 during OPC, with our hypothesis being that *Saa3*$^{-/-}$ (KO) mice will be more susceptible to OPC in comparison to WT-infected mice. Based on this, our initial objective was to characterize host innate immune responses during OPC in *Saa3*$^{-/-}$ (KO) mice to establish the mechanisms driving susceptibility to OPC. The second aim was to determine the role of SAA3 in regulation of neutrophils during OPC with our hypothesis being SAA3 regulates neutrophil recruitment during oral candidiasis.

Chapter 2

Materials and Methods

2.1 Cohorts of mice

The animal protocol was carried out in compliance with the University of Toledo's Institutional Animal Care Unit Committee (IACUC). The following mouse cohorts were used in this study: *IL17ra*$^{-/-}$, *Saa3*$^{+/+}$ (WT), and *Saa3*$^{-/-}$ (KO). As a result, *IL17ra*$^{-/-}$ mice are treated as positive controls since they lack the IL-17RA receptor which blocks IL-17 signaling, which makes them most sensitive to OPC infection (Conti & Gaffen, 2015) (Conti et al., 2009). Furthermore, cortisone-treated mice become very susceptible to OPC as they are immunosuppressed, also used as positive controls (Conti et al., 2009). *Saa3*$^{-/-}$ (KO) mice were created by the Knockout Mice Project (KOMP) Repository (Davis, CA) and kept on a C57/BL6 genetic background. We obtained the *Saa3*$^{+/+}$ (WT) and *Saa3*$^{-/-}$ (KO) mice from the University of Vermont for our investigations. All the littermates utilized in the experiments were 6-8 weeks old and were sex matched.

2.2 Preparation of overnight culture *Candida albicans*

C.albicans suspension was prepared in a shaker overnight at 30º C and 220 rpm using a single colony of *C. albicans* strain (SC5314) for 12-18 hours using Yeast Extract Peptone Dextrose (YPD) broth. After incubation, the *C. albicans* suspension had an OD_{600} of 1.2.

The altered suspension was then spun down, allowing the pellet to fall to the bottom. The supernatant was drained, and the pellet was resuspended in Phosphate Buffered Saline (PBS) solution.

2.3 Induction of OPC

All mice were weighed first, to track their percent weight loss. All the mice were anesthetized first based on their weight respectively. Before, infection oral pre-swabs were obtained by gently rubbing the swab over the tongues and on the YPD plates respectively to assess the presence of commensal fungi in the oral cavity of the mice used. OPC infection was then followed by using pre-weighed (0.0023g-0.0027g) cotton balls soaked in *C. albicans* suspension inserted sublingually inside the mouth of the mouse for 75 minutes. Throughout the experiment, all mice were monitored. Additionally, during infection, all mice were given 1 ml of 0.9% saline solution subcutaneously at the start and end of the infection to keep them hydrated. During the infection, eye ointment was used to keep the eyes moist. A heat lamp was also utilized to keep the cages warm and pleasant. Wild-type mice injected subcutaneously with cortisone acetate (225 mg/kg I.P.) were used as a positive control. Cortisone acetate was injected on alternative days -1, 1, and 3 during infection. Homogenization of tongue tissue was followed by plating serial dilutions on YPD plates with antibiotics to assess fungal load.

2.4 Quantification of Fungal Burden

Fresh tongue tissue samples were collected from all mice in the experiments, weighed, and homogenized by suspending in 500µl Phosphate Buffered Saline solution- 1xdPBS (for half tongues) and 2 ml 1x dPBS (for kidneys) using a Gentle MACS Dissociator from Miltenyi Biotech (Bergisch, Germany). On each YPD plate, 100µl of homogenate (suspension of tongue tissue fragments obtained) were added in triplicates and incubated at 30°C. After 48 hours of incubation, colonies were counted, and statistics were analyzed as colony-forming units per gram tongue tissue (CFU/g).

2.5 Flow Cytometry

Tongue samples were harvested on day two post-infection to look at different immune cell populations. Tongues were normally bigger in size, so they are cut into small 1-2 mm sized pieces, by placing into 2mL of RPMI 1640 media, followed by the addition of an enzyme mixture consisting of DNase, collagenase, and dispase from Miltenyi Biotec at 37°C for 42 minutes using GentleMACS Dissociator. The solution was passed through a 40µM filter, centrifuged at 1500rpm for 1min, followed by reconstitution in 1mL of a FACS Buffer solution to FACS tube. Cells were then stained with probes of interest such as GR1 (neutrophil marker), CD11b (generic granulocytic/monocytic marker), CD45 (generic leukocytic marker), and TCRb (IL-17 producing cells) conjugated with APC, FITC, PE-Cy7 respectively. The staining was carried out by incubating for 30 minutes on ice, followed by flow-analysis using the LSRFortessa. An unstained control was normally included using the $SSC^{+}FSC^{+}$ plot to determine the location of the negative population.

The $SSC^{+}FSC^{+}$ plot is directly proportional to cell granularity complex & cell diameter respectively. The collected data was then analyzed through FlowJo software.

2.6 RNA extraction, cDNA synthesis & Real-time qPCR analysis

Tongue samples of infected mice were harvested on respective days to analyze the genetic profile of that mice by RT-qPCR using primers from QIAGEN in triplicates using SYBR Green and normalizing to housekeeping gene control GAPDH. Each tongue sample was dissected into two halves with right halves being used for RNA processing (meanwhile stored at -80°C) using an RNA extraction kit from Applied Biosystems (Foster City, CA). The tissues were placed in a mixture of 500µL lysis buffer and then homogenized on the GentlMACS Dissociator for 2 minutes, followed by a 1ml 70% ethanol wash before pipetting the solution into a separate Eppendorf tube and centrifuging at 12,000-x g for 3 minutes. Three consecutive washes, each followed by a 12,000-x g centrifugation, were performed before the RNA is eluted into a final collection tube with 35µL of RNase-free water. Sample quality was determined by $OD_{260/280}$ and quantity measured in ng/µL, with OD of >1.8 and quantity >100ng/µL being considered ideal. cDNA synthesis was performed using an Applied Biosystems cDNA Reverse Transcription Kit, with all samples standardized to a quantity of 500ng/µL. Following a short incubation of all samples at room temperature in the presence of DNase, I for 5 minutes and then subsequent inactivation via incubation at 65°C for 10 minutes, a master mix containing Luna Superscript RT buffer, nuclease-free water, and RNA samples were added and then PCR performed. The resulting cDNA product was used to analyze gene transcripts in an Applied Biosystems Quant-

Studio 3 Real-Time qPCR instrument. For each qPCR reaction, 1x concentration of gene primer from Qiagen was added to a 100ng/µL concentration of cDNA sample along with SYBR Green master mix and run for a total of 40 cycles to obtain relevant Ct values. GAPDH was utilized as the housekeeping gene as they do not change the relative expression value with treatments. Each experiment should be repeated at least three times.

2.7 Histology

Tongue tissue samples were stained with H&E (Hematoxylin and Eosin) for histological analysis to assess the presence of neutrophils. The tongue samples were kept in 10% formalin overnight after harvesting, followed by ethanol washes for dehydration, and ultimately embedded in paraffin blocks for tissue sectioning and staining. Neutrophils were counted in areas of the tongue affected by the fungal invasion based on imaged slides taken on the Evos microscope at various magnifications (Saul-McBeth et al., 2021).

2.8 Data Analysis

ANOVA and Mann-Whitney non-parametric t-test were used to statistically analyze the data using GraphPad Prism software. We mostly used ANOVA here to compare the means of three or more independent groups as it includes a common P-value. A significant P value in the ANOVA test implies a $P<0.05$ with a single star (*) whereas $P<0.01$, $P<0.001$, and $P<0.0001$ are denoted with two stars (**), three stars (***) and four stars (****) respectively.

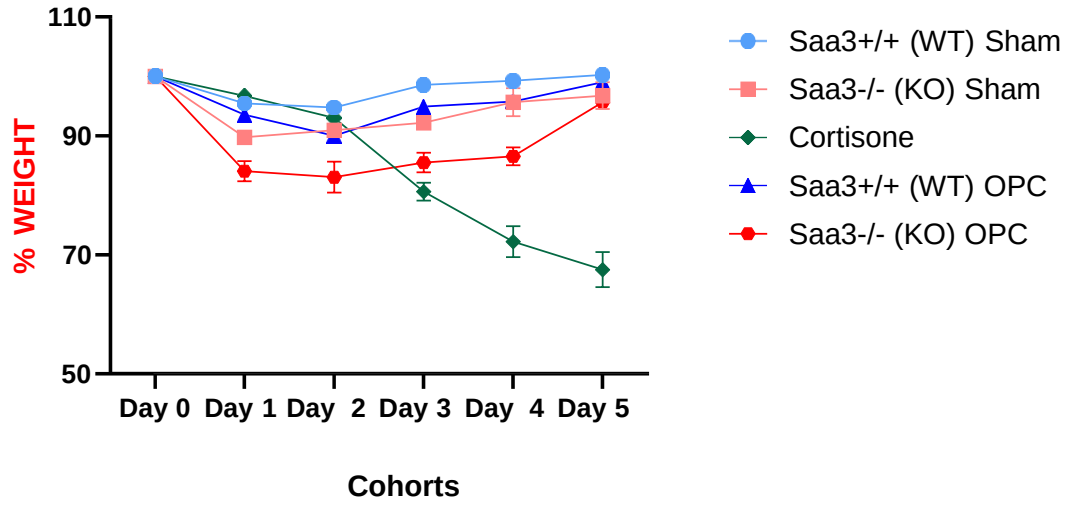
Chapter 3

Results

3.1 Susceptibility to OPC infection in *Saa3*$^{-/-}$ (KO) mice

To study the importance of SAA3 during fungal pathogenesis, *Saa3*$^{-/-}$ (KO) mice were infected with *Candida*, and their fungal burden (CFU/g tongue tissue) were compared to *Saa3*$^{+/+}$ WT (wild type) mice (Fig 3.1 A&B). Utilizing our well-established model of OPC infection following the day-5 protocol, sham mice treated as negative controls, cortisone-treated mice as positive controls and *Saa3*$^{+/+}$ (WT) & *Saa3*$^{-/-}$ (KO) mice as experimental groups were analyzed. However, on day 5 we observed no significant difference between *Saa3*$^{+/+}$ (WT) and *Saa3*$^{-/-}$ (KO) mice as both cohorts regained 100% weight with no fungal burden detectable. This led us to hypothesize that maybe the time point of clearing infection was early in Saa3 mice compared to other WT mice.

A



B

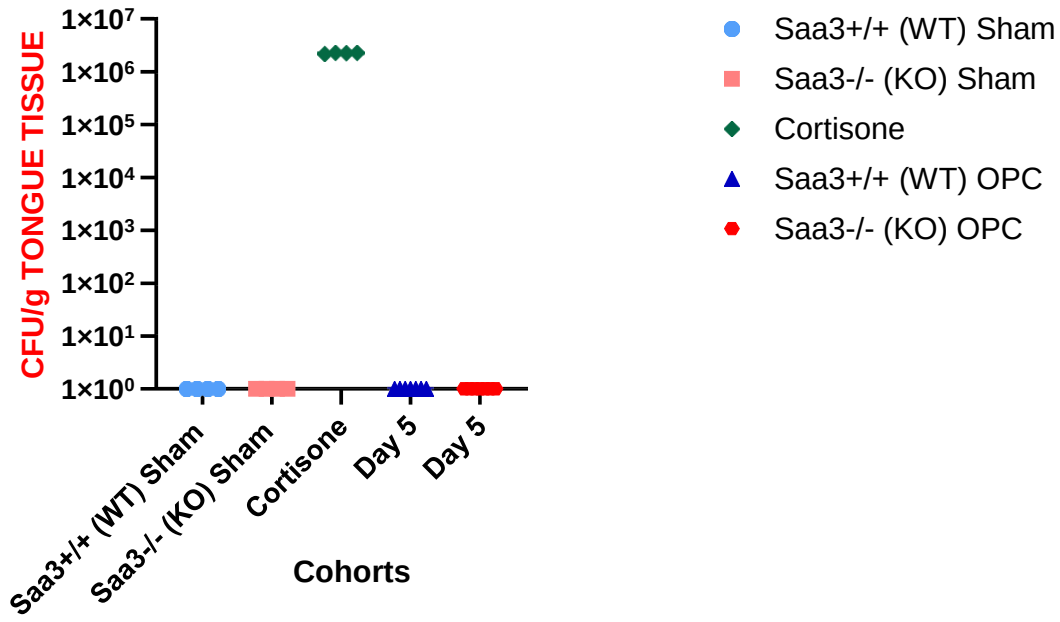
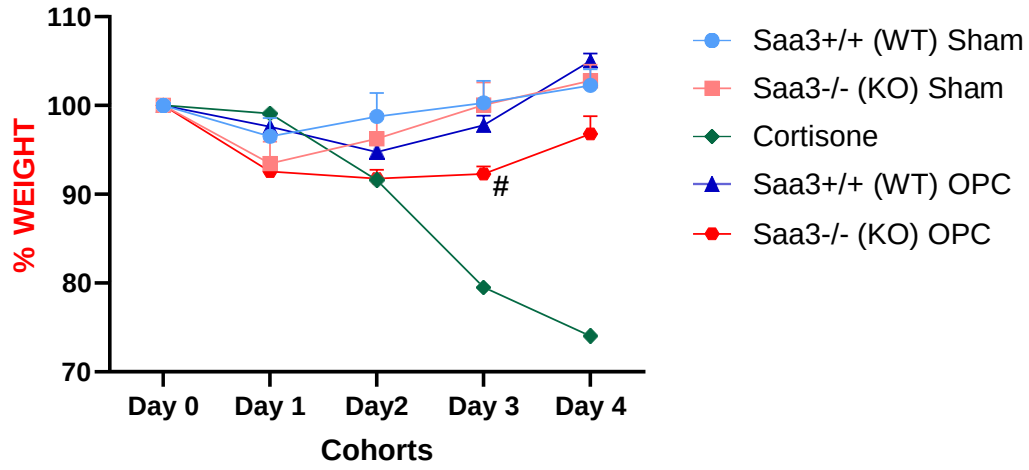


Figure 3.1- A & B: *In vivo* OPC model and analysis of body weight and fungal burden. A. Percent weight loss on respective days. **B.** Tongue tissue was harvested on day 5 post infection and plated on YPD agar to determine fungal burden by colony forming units per gram of tongue tissue (CFU/g) where both *Saa3*$^{+/+}$ (WT) and *Saa3*$^{-/-}$ (KO) mice cleared the fungal burden. Data showed is a representative of three total experiments. Statistical analysis was performed using ANOVA with *Saa3*$^{+/+}$ (WT) Sham n=3, *Saa3*$^{-/-}$ (KO) Sham n=3, Cortisone n= 4, *Saa3*$^{+/+}$ (WT) OPC n= 6-7, *Saa3*$^{-/-}$ (KO) OPC n=6-7, where (n= total number of mice in each group used in this experiment).

C



D

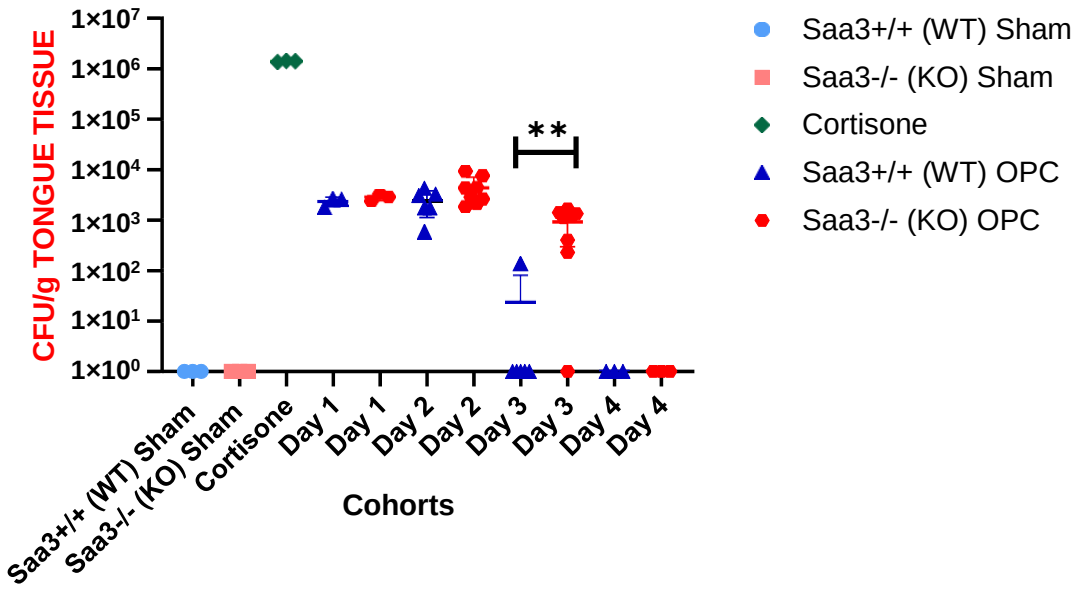


Figure 3.1- C&D: Susceptibility of *Saa3*$^{-/-}$ (KO) mice during OPC C. Percent weight loss on respective days **D.** Tongue tissue was harvested on day 1-4 post infection and plated on YPD agar to determine fungal burden by colony forming units per gram of tongue tissue (CFU/g) where *Saa3*$^{-/-}$ (KO) mice showed constant fungal burden on day 3 compared to WT mice. Data showed is a representative of three total experiments. Statistical analysis was performed using ANOVA, **P<0.01, # = **P<0.01 with *Saa3*$^{+/+}$ (WT) Sham n=3, *Saa3*$^{-/-}$ (KO) Sham n=3, Cortisone n= 3, *Saa3*$^{+/+}$ (WT) OPC n= 12-13, *Saa3*$^{-/-}$ (KO) OPC n=15-17, where (n= total number of mice in each group used in this experiment).

To test this hypothesis, we did a time-course analysis of fungal burden with *Saa3*$^{-/-}$ (KO) mice. Similar to the day-5 protocol, we opted to follow up the experiment by harvesting tongue tissues on days 2, 3 & 4 respectively (Fig 3.1 C&D) to assess the clearance of the infection in *Saa3*$^{-/-}$ (KO) mice. These mice were euthanized on respective days and fungal burden (CFU per gram tongue tissue) was assessed. We observed that in *Saa3*$^{+/+}$ (WT) mice, there was almost 95% recovery of weight, and clearance of fungal burden by day 3 whereas *Saa3*$^{-/-}$ (KO) mice significantly lost more body weight with defects in fungal clearance on day 3 post-infection. The experiment was conducted three times, as a result, it alludes to the importance of Saa3 having a protective role during OPC.

3.2 Reduced expression of *Saa1* and *Saa2* genes in absence of *Saa3* during oral *Candida* infection

The important role of SAA3 has already been demonstrated in previous studies, where SAA3 showed the importance in normal lung development and protection against influenza infection with elevated levels of IL-17 production (Ather et al., 2018). Based on published data, *Saa1* and *Saa2* levels were not found to be compensated in the lung despite the absence of *Saa3* (Ather et al., 2018). However, in this experiment as we found a clearance of fungal burden in *Saa3*$^{-/-}$(KO) mice by day 5, we hypothesize to check other SAA family members (Saa1 & Saa2) who might have a role in clearing fungal infection at that time point. The initial investigation involves the expression of *SAA* genes (such as *Saa1 & Saa2*) at early time points which were found to be lower on day 2 post-infection in both *Saa3* WT-OPC and *Saa3*$^{-/-}$ (KO) mice. However, on day 3 post-infection in *Saa3*$^{+/+}$ OPC mice the expression was markedly increased, but in *Saa3*$^{-/-}$ (KO) mice it was

significantly impaired, implying that SAA genes (*Saa1 & Saa2*) are not compensating for the lack of Saa3 in *Saa3*$^{-/-}$ (KO) mice in clearing fungal infection (Fig 3.2 A & B).

A

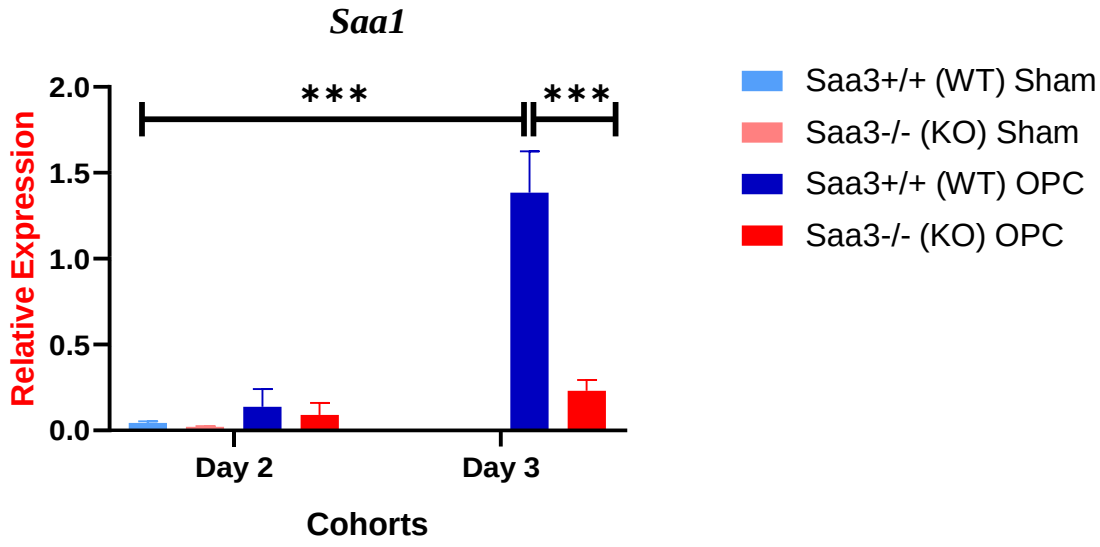


Figure 3.2A: Gene expression changes during OPC show expression of *Saa1* in *C. albicans* infected *Saa3*$^{+/+}$ (WT) but not in *Saa3*$^{-/-}$ (KO) mice on day 3 post-infection. RNA extracted from tongue tissue harvested from *C. albicans* infected mice and control (Sham) was used for gene expression studies. The data shown are representative of three total experiments. Statistical analysis was performed using ANOVA, ***P<0.001 with (qPCR, n=3-5) D2-D3= Days post-infection where (n= total number of mice in each group used in this experiment).

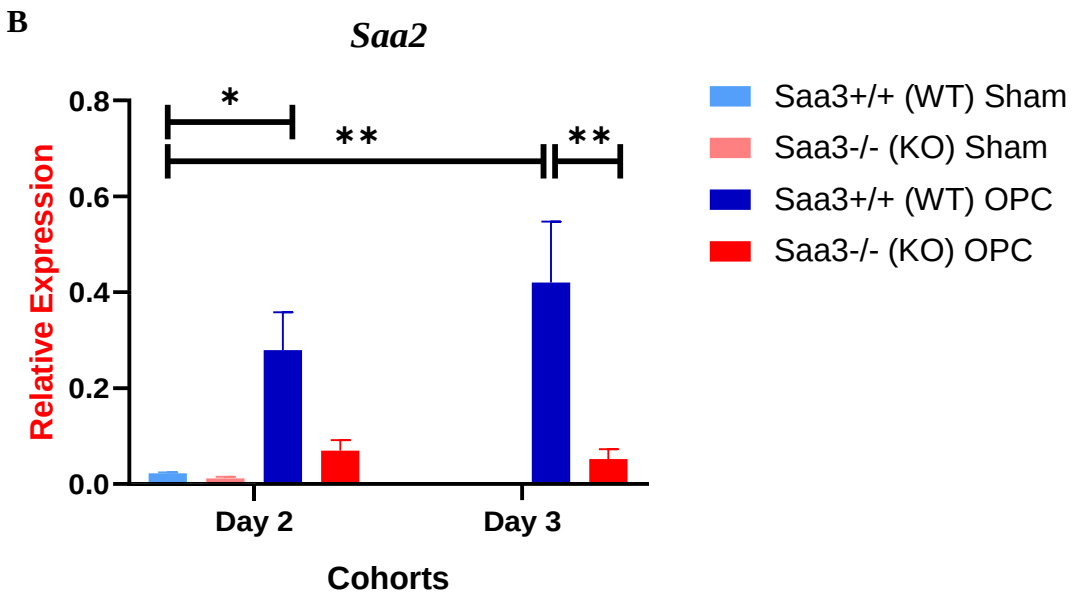


Figure 3.2B: Gene expression changes during OPC show expression of *Saa2* in *C. albicans* infected *Saa3*$^{+/+}$ (WT) but not in *Saa3*$^{-/-}$ (KO) mice on day 3 post-infection. RNA extracted from tongue tissue harvested from *C. albicans* infected mice and control (Sham) was used for gene expression studies. The data shown are representative of three total experiments. Statistical analysis was performed using ANOVA, *P<0.05, **P<0.01 with (qPCR, n=3-5) D2-D3= Days post-infection where (n= total number of mice in each group used in this experiment).

3.3 Effect of knocking out *Saa3* on antifungal genes during OPC

The critical significance of IL-17 in *Candida* clearance during OPC has long been recognized (Conti et al., 2009) (Conti et al., 2016), and as we have not identified any important role of *Saa1* and *Saa2* in *Saa3-/-* (KO) OPC mice we intended to study the other gene expression pattern of *Il17, Il6, TGFβ, Tnfa, Il1β,* and AMPs (*Defb3 and S100A9*) who are known to have antifungal effect during OPC. The initial investigation includes low *Il17a* expression in both $Saa3^{+/+}$ Sham and $Saa3^{-/-}$ (KO) Sham. Initially, *Il17a* was strongly induced in $Saa3^{+/+}$ OPC (day 2&3) compared to *Saa3-/-* (KO) OPC which explains why *Saa3-/-* (KO) OPC mice have delayed fungal clearance. However, the *Il17a* expression level was suddenly seen increased in $Saa3^{-/-}$ OPC mice at day 4 compared to $Saa3^{-/-}$ (KO) Sham suggesting its activity of antifungal immune response during OPC lately (Fig 3.3 A).

Similarly, *Il1β* expression was reduced in *Saa3-/-* (KO) OPC (day 2&3 post-infection) compared to $Saa3^{+/+}$ mice. However, the expression of *Il1β* in *Saa3-/-* (KO) OPC was seen to be increased on day 4 with a significant difference with *Saa3-/-* (KO) Sham (Fig 3.3 B). Moreover, the gene expression pattern of *Il6* also showed a similar pattern that it was strongly induced in only $Saa3^{+/+}$ OPC (day 2 &3 post-infection). However, the expression of *Il6* was consistently reduced in $Saa3^{-/-}$ (KO) OPC at day 2& 3 post-infection, but by day 4, it got dramatically increased, implying its role in the reduction of antifungal immunity at early time points (Fig 3.3 C).

A

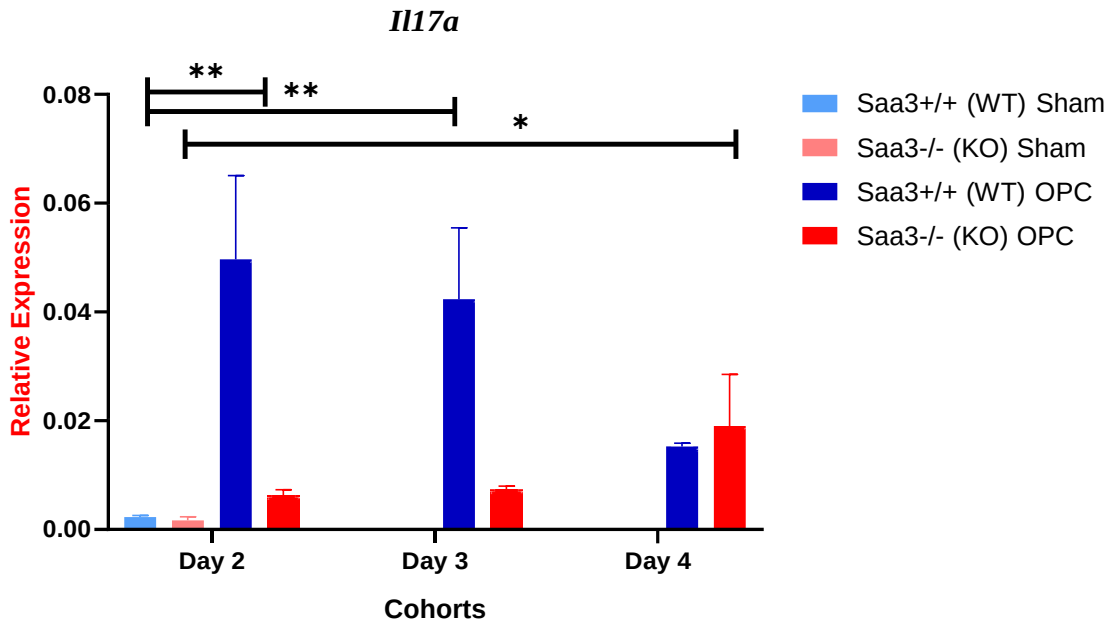


Figure 3.3A: Induction of *Il17a* gene expression during OPC in *C. albicans* infected *Saa3*$^{-/-}$ (KO) mice on day 4 post-infection. RNA extracted from tongue tissue harvested from *C. albicans* infected mice and control (Sham) was used for gene expression studies. The data shown are representative of three total experiments. Statistical analysis was performed using ANOVA, *P<0.05, **P<0.01 with (qPCR, n=3-5) D2-D4= Days post-infection where (n= total number of mice in each group used in this experiment).

B

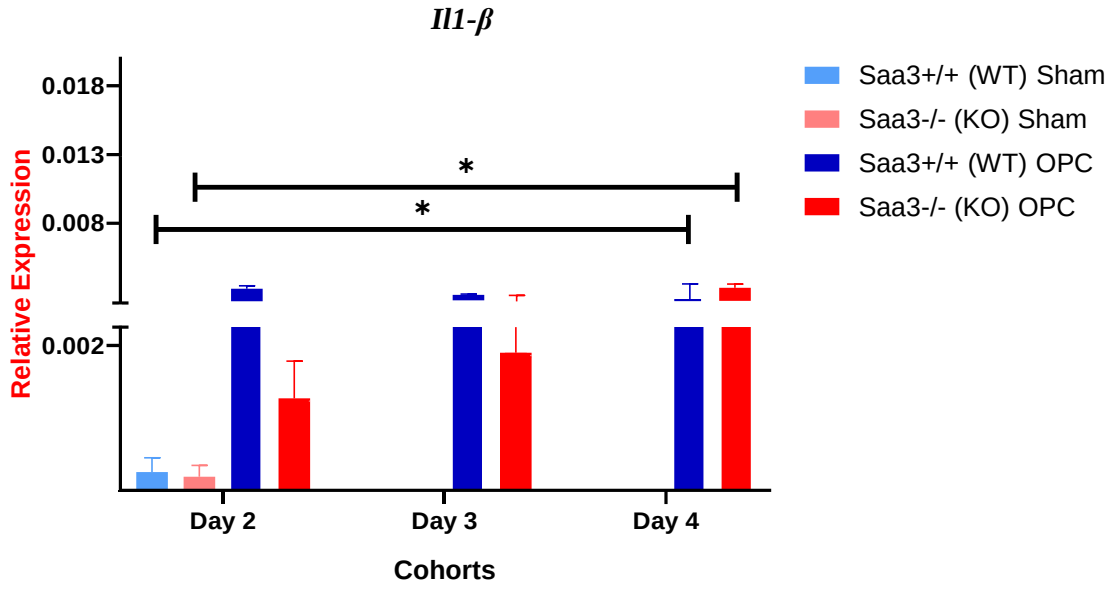


Figure 3.3B: *Il1β* gene expression during OPC in *C. albicans* infected *Saa3*$^{-/-}$ (KO) mice on day 4 post-infection. RNA extracted from tongue tissue harvested from *C. albicans* infected mice and control (Sham) was used for gene expression studies. The data shown are representative of three total experiments. Statistical analysis was performed using ANOVA, *P<0.05 with (qPCR, n=3-5) D2-D4= Days post-infection where (n= total number of mice in each group used in this experiment).

C

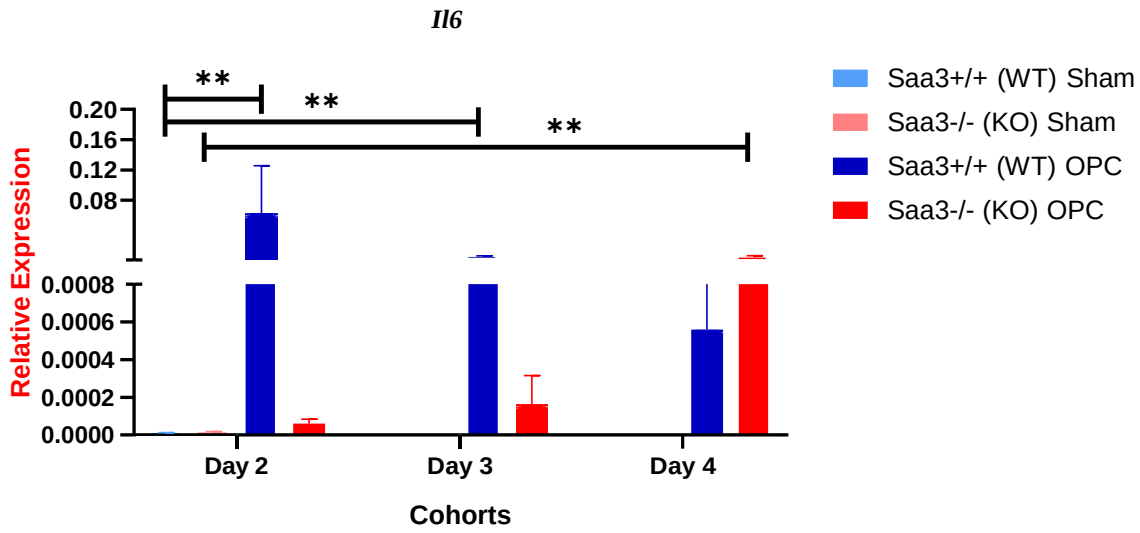


Figure 3.3C: Expression of *Il6* gene in *C. albicans* infected in *Saa3*$^{-/-}$ (KO) mice on later time point (day 4 post-infection). RNA extracted from tongue tissue harvested from *C. albicans* infected mice and control (Sham) was used for gene expression studies. The data shown are representative of three total experiments. Statistical analysis was performed using ANOVA, **P<0.01 with (qPCR, n=3-5) D2-D4= Days post-infection where (n= total number of mice in each group used in this experiment).

Similarly, *TGFβ* was significantly seen reduced initially (day 2& 3 post-infection) in *Saa3-/-* (KO) OPC (Fig 3.3 D) consistent with its function in the antifungal immune response during OPC.

Studies include other downstream antifungal cytokines such as interleukin 1 (IL-1) which plays an important function during OPC infection. Similarly, we observed expression of *Il1a* in $Saa3^{+/+}$ (WT) OPC but decreased in $Saa3^{-/-}$ (KO) OPC on days 2 and 3. However, the expression increased significantly in $Saa3^{-/-}$ (KO) OPC on day 4 post-infection in comparison to $Saa3^{-/-}$ (KO) Sham, showing its critical role in host defense in regulating neutrophil response at later time points (Fig 3.3 E). Similarly, *Tnfa* expression showed strong induction in $Saa3^{+/+}$ (WT) mice compared with their respective sham (non-infected) controls. However, overall *Tnfa* was seen reduced in $Saa3^{-/-}$ (KO) OPC mice at early time points except at day 4 where the expression got increased (Fig 3.3 F). This further explains why $Saa3^{-/-}$ (KO) OPC mice have a high fungal burden at early time points. We also studied the gene expression patterns of selected genes like murine *β-Defensin 3* genes (*Defb3*) and neutrophil-associated proteins (*S100A9*) to better understand how SAA3 contributes to OPC immunity. However, we observed expression of *Defb3* in both $Saa3^{+/+}$ (WT) OPC and $Saa3^{-/-}$ (KO) OPC mice on days 3 & 4 post-infection compared to Sham controls which shows its activity towards antifungal immune response at that time point (Fig 3.3 G). On the other hand, the reduced expression of *S100A9* observed in $Saa3^{-/-}$ (KO) OPC mice throughout (Fig 3.3 H), implies no direct correlation in the immune response in presence of Saa3.

D

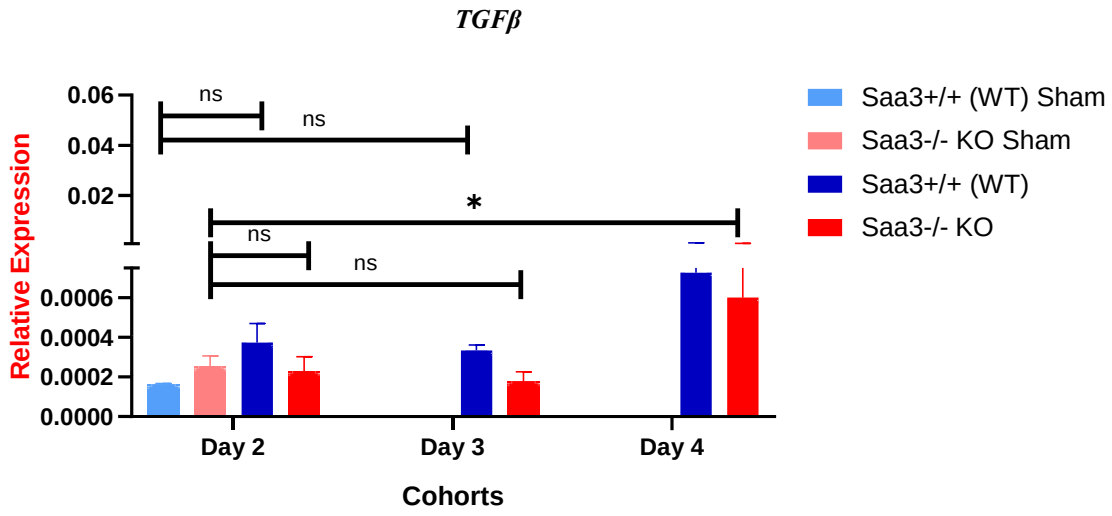


Figure 3.3D: *TGFβ* gene expression changes during OPC in *C. albicans* infected *Saa3*$^{-/-}$ (KO) mice on day 4 post-infection. RNA extracted from tongue tissue harvested from *C. albicans* infected mice and control (Sham) was used for gene expression studies. The data shown are representative of three total experiments. Statistical analysis was performed using ANOVA, *P<0.05, ns= statistically not significant at (P< 0.05) with (qPCR, n=3-5) D2-D4= Days post-infection where (n= total number of mice in each group used in this experiment).

E

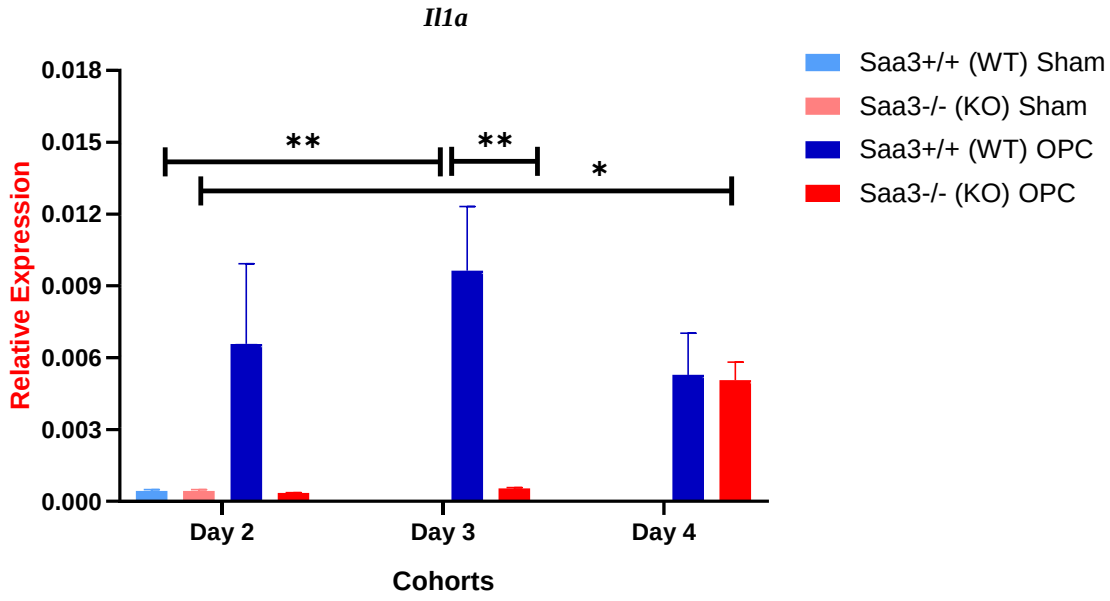


Figure 3.3E: *Il1a* gene expression during OPC in *C. albicans* infected *Saa3*$^{-/-}$ (KO) mice on day 4 post-infection. RNA extracted from tongue tissue harvested from *C. albicans* infected mice and control (Sham) was used for gene expression studies. The data shown are representative of three total experiments. Statistical analysis was performed using ANOVA, *P<0.05, **P<0.01 with (qPCR, n=3-5) D2-D4= Days post-infection where (n= total number of mice in each group used in this experiment).

F

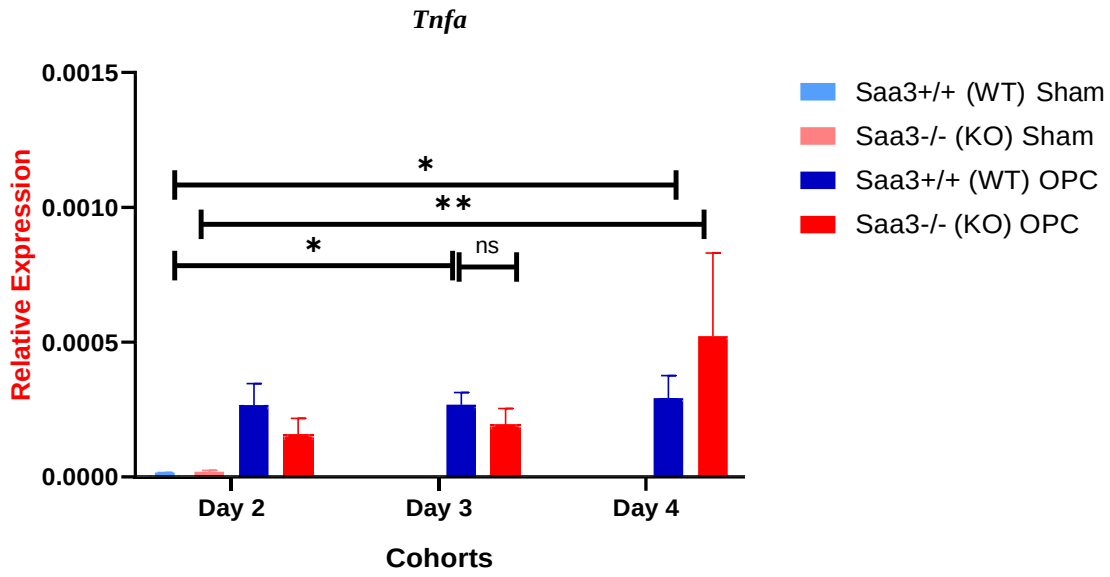


Figure 3.3F: *Tnfa* gene expression changes during OPC in *C. albicans* infected *Saa3*$^{-/-}$ (KO) mice at day 4 post-infection. RNA extracted from tongue tissue harvested from *C. albicans* infected mice and control (Sham) was used for gene expression studies. The data shown are representative of three total experiments. Statistical analysis was performed using ANOVA, *P<0.05, **P<0.01, ns= statistically not significant at (P< 0.05) with (qPCR, n=3-5) D2-D4= Days post-infection where (n= total number of mice in each group used in this experiment).

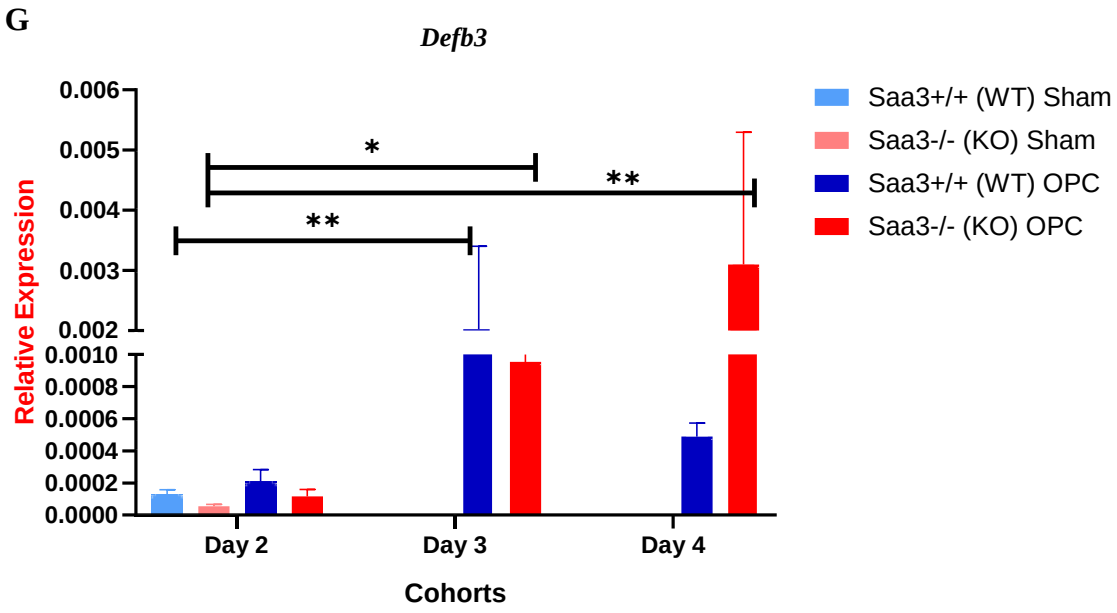


Figure 3.3G: *Defb3* gene expression during OPC in *C. albicans* infected *Saa3*$^{-/-}$ (KO) mice on days 3 & 4 post-infection. RNA extracted from tongue tissue harvested from *C. albicans* infected mice and control (Sham) was used for gene expression studies. The data shown are representative of three total experiments. Statistical analysis was performed using ANOVA, *P<0.05, **P<0.01 with (qPCR, n=3-5) D2-D4= Days post-infection where (n= total number of mice in each group used in this experiment).

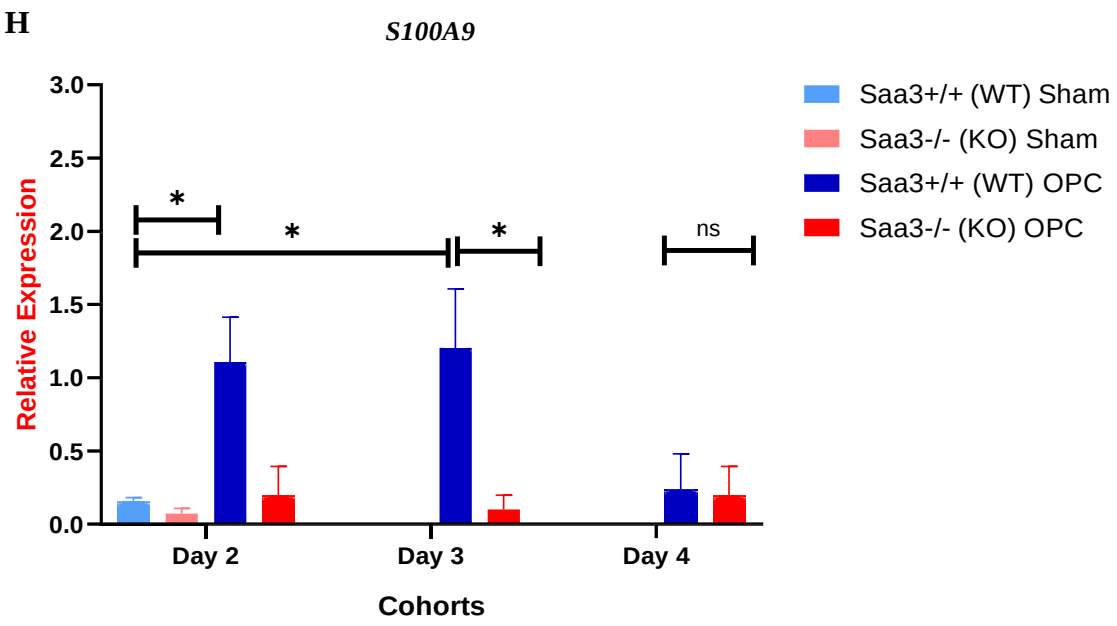


Figure 3.3H: Reduced *S100A9* gene expression during OPC in *C. albicans* infected *Saa3*$^{-/-}$ (KO) mice on days 2, 3 & 4 post-infection respectively. RNA extracted from tongue tissue harvested from *C. albicans* infected mice and control (Sham) was used for gene expression studies. The data shown are representative of three total experiments. Statistical analysis was performed using ANOVA, *P<0.05, ns= statistically not significant at (P< 0.05) with (qPCR, n=3-5) D2-D4= Days post-infection where (n= total number of mice in each group used in this experiment).

Additionally, further downstream includes neutrophil chemokines *Cxcl1* and *Cxcl2* which were also found to be significantly impaired in *Saa3*$^{-/-}$ (KO) OPC mice compared with *Saa3*$^{+/+}$ (WT) OPC mice consistent with the defects in neutrophils recruitment found in *Saa3*$^{-/-}$ (KO) OPC mice discussed later. However, the expression of *Cxcl5* was found to be comparable in both *Saa3*$^{+/+}$ (WT) OPC and *Saa3*$^{-/-}$ (KO) OPC mice (Fig 3.3 I, J & K).

I

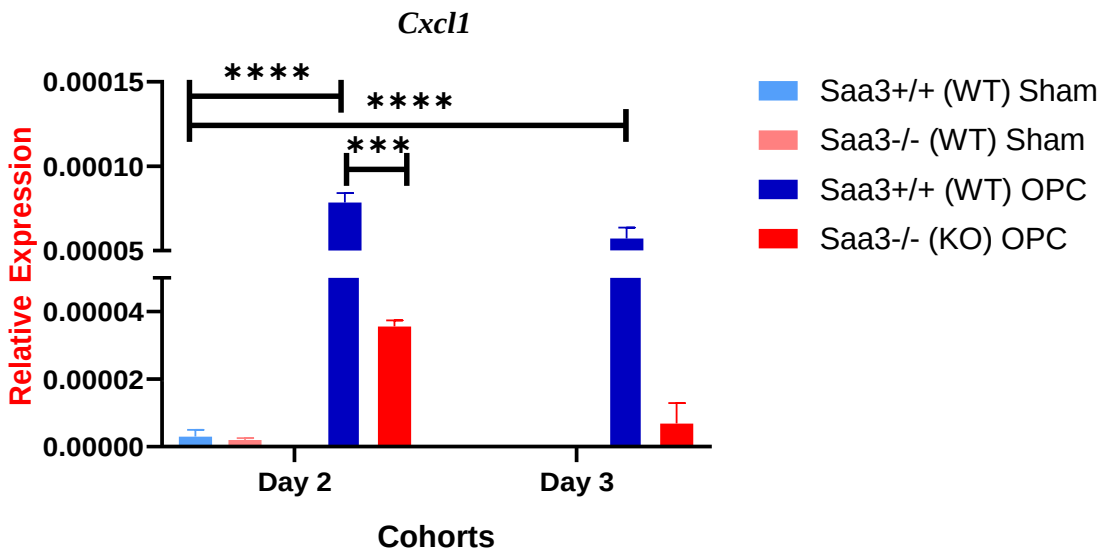


Figure 3.3I: *Cxcl1* expression reduced during OPC in *C. albicans* infected *Saa3*$^{-/-}$ (KO) mice on day 3 post-infection. RNA extracted from tongue tissue harvested from *C. albicans* infected mice and control (Sham) was used for gene expression studies. The data shown are representative of three total experiments. Statistical analysis was performed using ANOVA, ****P<0.0001, ***P<0.001 with (qPCR, n=3-5) D2-D3= Days post-infection where (n= total number of mice in each group used in this experiment).

J

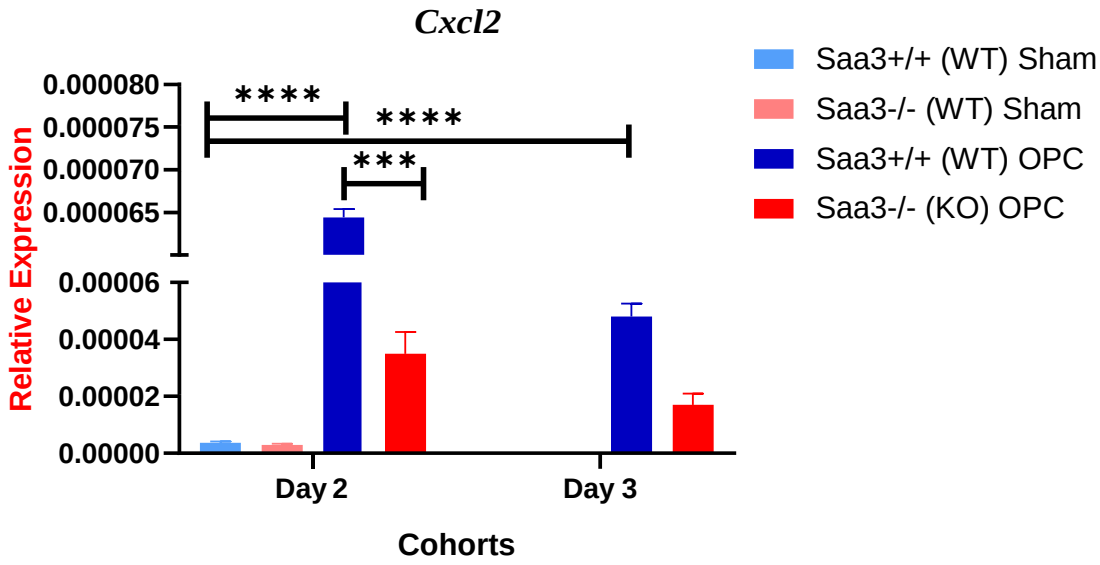


Figure 3.3J: *Cxcl2* expression reduced during OPC in *C. albicans* infected *Saa3*$^{-/-}$ (KO) mice on day 3 post-infection. RNA extracted from tongue tissue harvested from *C. albicans* infected mice and control (Sham) was used for gene expression studies. The data shown are representative of three total experiments. Statistical analysis was performed using ANOVA, ****P<0.0001, ***P<0.001, and ns= statistically not significant at (P< 0.05) with (qPCR, n=3-5) D2-D3= Days post-infection where (n= total number of mice in each group used in this experiment).

K

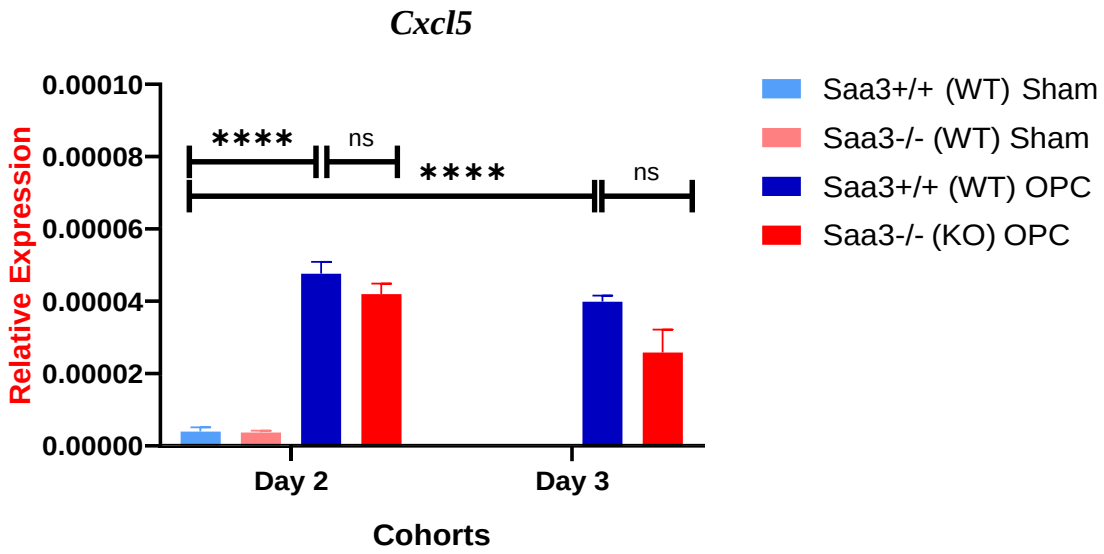


Figure 3.3K: *Cxcl5* expression comparable during OPC in both *C. albicans* infected *Saa3*$^{+/+}$ (WT) and *Saa3*$^{-/-}$ (KO) mice on day 2 & 3 post-infection. RNA extracted from tongue tissue harvested from *C. albicans* infected mice and control (Sham) was used for gene expression studies. The data shown are representative of three total experiments. Statistical analysis was performed using ANOVA, ****P<0.0001, and ns= statistically not significant at (P< 0.05) with (qPCR, n=3-5) D2-D3= Days post-infection where (n= total number of mice in each group used in this experiment).

3.4 Immunohistochemical analysis show defects in neutrophil recruitment in *Saa3*$^{-/-}$ (KO) mice undergoing OPC

As we observed reduced expression of *Cxc1*, *Cxl2* & *Cxcl5* in *Saa3*$^{-/-}$ (KO) OPC mice at day 2 & 3-time points, we wanted to determine the role of SAA3 in the regulation of neutrophils during OPC. Therefore, to assess neutrophils in *Saa3* deletion and WT control mice, tongues were harvested on days 2 and 3 post-infection as neutrophils play a crucial role in controlling fungal infection (Conti et al., 2016). After harvesting tongue tissues were placed in 10% formalin overnight, followed by successive ethanol wash for dehydration, and finally embedded in paraffin blocks for tissue sectioning and staining. Hematoxylin and Eosin (H&E) stained tongue tissue sections were evaluated for the presence of neutrophils in supra and sub-basal regions of the tongue tissues (Fig 3.6 A).

The neutrophil recruitment was assessed between *Saa3*$^{+/+}$ (WT) OPC and *Saa3*$^{-/-}$ (KO) OPC mice comparing Sham as negative controls. The results of tissue imaging showed that the number of neutrophils in supra-basal regions between *Saa3*$^{+/+}$ (WT) OPC and *Saa3*$^{-/-}$ (KO) OPC mice were different significantly with distinct hyperplasia (commonly observed on the tongue, palate) on day 2 post-infection. However, by day 3, the overall number of neutrophils was slightly reduced in both the cohorts but in *Saa3*$^{+/+}$ (WT) OPC mice neutrophils were still persistent in both supra and sub basal areas with significant differences compared to *Saa3*$^{-/-}$ (KO) mice (Fig 3.6 B). Overall, this indicates that loss of *Saa3* in mice undergoing OPC shows defects in neutrophil recruitment to the site of infection.

A

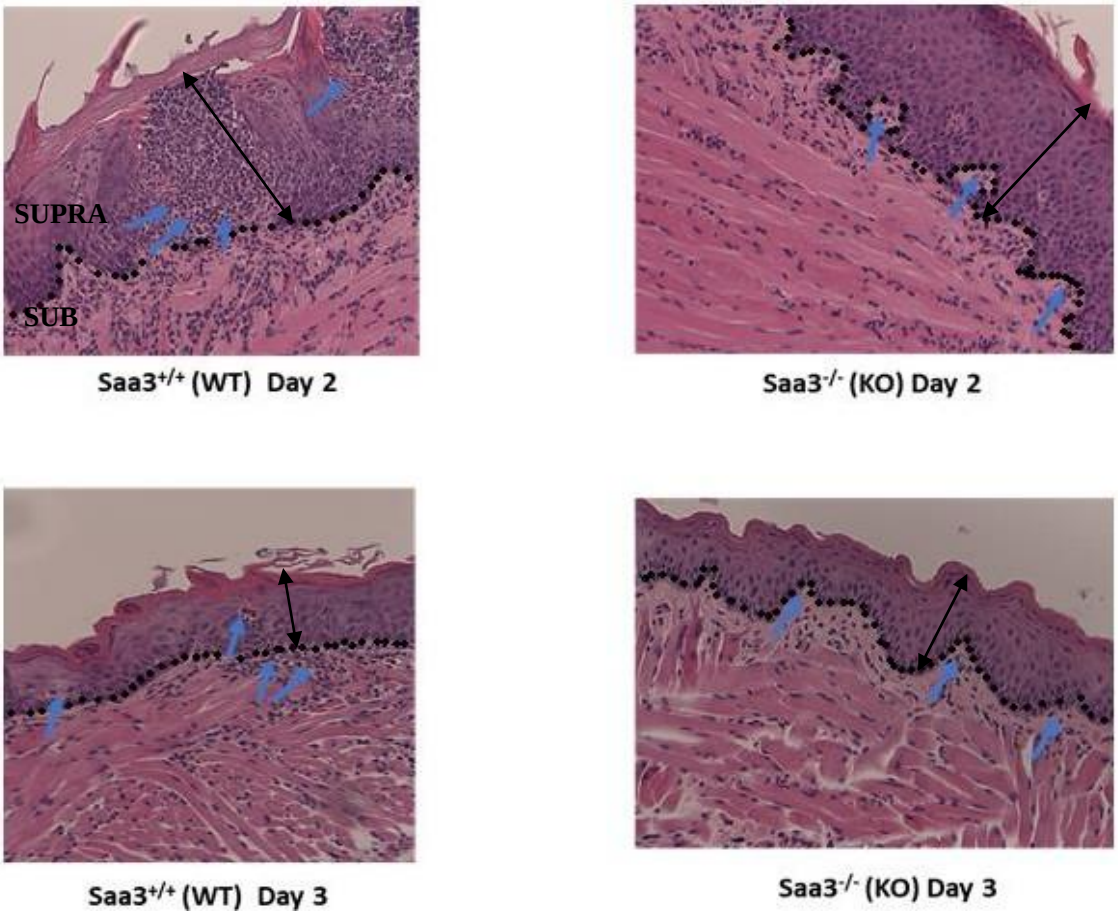


Figure 3.4A: Defects in neutrophil recruitment in *Saa3*$^{-/-}$ (KO) mice during OPC. Representative sections of each cohort *Saa3*$^{+/+}$ (WT) and *Saa3*$^{-/-}$ (KO) with different layers (supra and sub basal layers) are indicated. The tissue section was stained with H&E, imaged at 20x magnification, and neutrophils were counted together where **blue arrows** indicate neutrophils, **black arrows** indicate hyperplasia, **black dots** indicate the basal stem cell layer and the **upper pink border** indicating the supra-basal region and **the lower light pink portion** the sub-basal region.

B

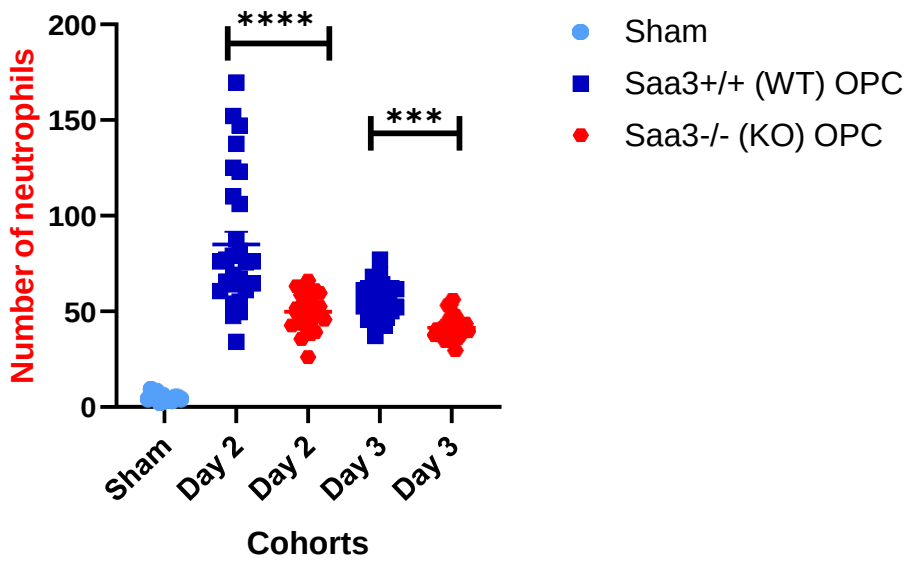


Figure 3.4B: Neutrophils were separately counted between each cohort on days 2&3 post-infection. *Saa3*$^{+/+}$ (WT) and *Saa3*$^{-/-}$ (KO) mice undergoing OPC show a significant difference on days 2&3 post-infection Statistical analysis was performed using ANOVA by counting neutrophils present in different layers (supra and sub basal layers together) ****P<0.0001 and ***P<0.001, D2-D3= Days post-infection respectively.

3.5 Immune cells population changes in *Saa3*$^{-/-}$ (KO) mice undergoing OPC

To further support our study, we analyze different innate immune cells that are important in the oral cavity during OPC using flow cytometry. To assess cell recruitment during OPC infection (Figure3.5A, B & C) both *Saa3*$^{-/-}$ (KO) and WT litter mates were infected with *C. albicans* along with their respective Sham treated as controls. Mice were euthanized 2 days after infection, and their tongues were collected and processed for flow cytometric analysis. Cells were stained with probes of interest such as GR1 (neutrophil marker), CD11b (generic granulocytic/monocytic marker), CD45 (generic leukocytic marker), and TCRβ (IL-17 producing cells) conjugated with APC, FITC, PE-Cy7 respectively (Figure 3.5A). The data was analyzed using the SSC+FSC+ plot which is directly proportional to cell granularity complex & cell diameter respectively. Additionally, an unstained control was normally included to determine the location of the negative population (Figure 3.5A).

Comparing both WT and *Saa3*$^{-/-}$ (KO) mice it was observed that the overall percentage of all these populations were expanded in WT mice however in *Saa3*$^{-/-}$ (KO) mice it got significantly reduced including neutrophils, suggesting that there is a unique population shift between WT and *Saa3*$^{-/-}$ (KO) mice after OPC infection (3.5B, C & D). Overall, the data suggest that a lack of *Saa3* impairs immune cell population including neutrophil recruitment to the site of infection.

A

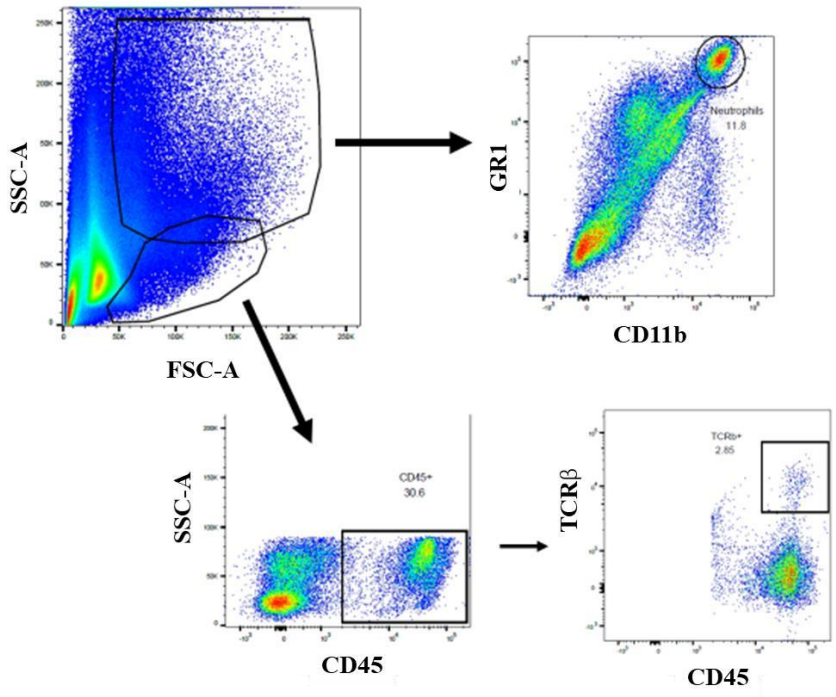


Figure 3.5A: Live gated using SSC+FSC+ plot shows cells positive for GR1, CD11b, CD45, and TCRβ conjugated with APC, FITC, PE-Cy7 respectively. Both *Saa3*$^{+/+}$ (WT) and *Saa3*$^{-/-}$ (KO) mice were subjected to OPC infection. Mice were euthanized 2- day post-infection and their tongues were harvested (WT-Sham n=3, *Saa3*$^{-/-}$ (KO) Sham n=3, WT-OPC n=3, and *Saa3*$^{-/-}$ (KO) OPC n=3) where 'n' denotes total number of mice in each group used in this experiment.
(Launder, Dylan)

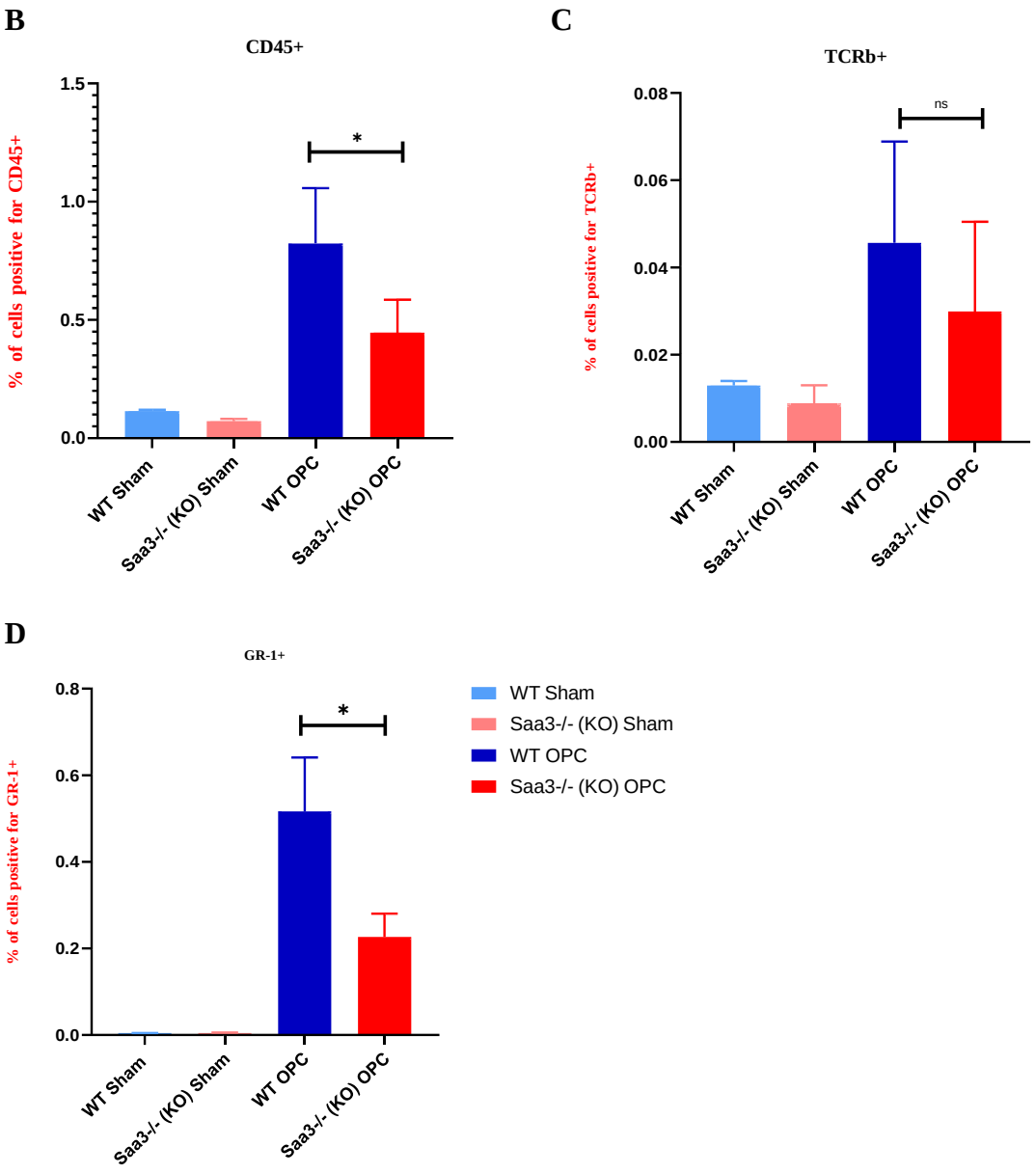


Figure 3.5B, C & D: Changes in recruitment of cells positive for CD45+, TCRβ+ and GR1+ in absence of *Saa3* during OPC infection. Indicated mice were subjected to OPC infection. Statistical analysis was performed using ANOVA, at *P<0.05. Mice were euthanized 2- day post-infection and their tongues were harvested (WT-Sham n=3, *Saa3*$^{-/-}$ (KO) Sham n=3, WT-OPC n=3, and *Saa3*$^{-/-}$ (KO) n=3) where 'n' denotes total number of mice in each group used in this experiment. (Launder, Dylan)

Chapter 4

Discussion

Candida albicans (C. albicans) is a common commensal fungus that exists on the skin and inside the GI tract, mouth, and vagina without causing any pathological manifestations (Conti & Gaffen, 2015). However, *C. albicans* can become pathogenic and are responsible for a mucosal form of oropharyngeal candidiasis (OPC) in immunocompromised patients with HIV/AIDS, diabetes, and those who have had head-neck irradiation (Conti & Gaffen, 2015). During OPC infection, animals lacking the IL-17RA (*IL17ra*$^{-/-}$) component are unable to develop an immune response and eliminate infection as compared to WT mice (Conti et al., 2009). This emphasizes the importance of IL-17 signaling in the oral cavity via this receptor in the host's antifungal defense. This cytokine IL-17 signaling pathway is involved in the production of neutrophils, expression of the chemokines, and antimicrobial peptides like β-defensin 3, which provide robust protection against fungal infections (Mengesha & Conti, 2017) (Conti & Gaffen, 2015) (Lee et al., 2009). Similar to other AMPs with known antibacterial effects, many other proteins have similar expression patterns with pro-inflammatory properties which are found to be produced excessively during inflammation and infection (Zhang et al., 2021). One family of these proteins includes acute-phase proteins. The SAA family consists of many apolipoproteins that are primarily synthesized in the liver (Zheng et al., 2020). During injury, infection, or trauma, acute phase SAAs (A-SAA) are greatly elevated

(Eckersall et al., 2001). The four forms of A-SAAs are SAA1, SAA2, SAA3, and SAA4 (Ather et al., 2018). SAA1 & SAA2 have been an area of interest due to their antibacterial effect, and as they are known to be 69% identical to mouse SAA3, suggests using this as a model of human-related diseases. Many previous studies explained the protective role of SAA3 against bacterial & viral infections due to its antibacterial properties (Fan et al., 2020). Moreover, one common feature of fungi and bacteria is the cell wall. Therefore, studying the effect of SAA3 in respect to fungal infection (*in-vivo*) was exciting. Thus, we studied the role of SAA3 during OPC *in-vivo* to understand whether they have any role towards anti-Candida immunity.

Moreover, in the instance of OPC infection in WT mice, *Saa3* expression was found to be the most prevalent gene expressed that further supports the previous analysis of *Conti et al*. where there was a high induction of *Saa3* observed in *Candida albicans* infected mice. Additionally, when $Saa3^{-/-}$ (KO) mice were put through the OPC infection model for their fungal susceptibility, they demonstrated more loss of body weight significantly with a high fungal burden on day 3 post-infection compared with $Saa3^{+/+}$ (WT) OPC (Fig 3.1 C&D), suggesting the involvement of SAA3 in protection against OPC. This constant high fungal burden of $Saa3^{-/-}$ (KO) at day 3 post-infection suggests that SAA3 plays a role in OPC protection but at early time points. However, the observed similarity of fungal loads (CFU/g) between $Saa3^{-/-}$ (KO) OPC and $Saa3^{+/+}$ (WT) OPC during the first two days could be attributed to the protection conferred by the innate immune response. However, $Saa3^{-/-}$ (KO) mice at later time points (day 4) were observed to clear the fungal load. As a result, we hypothesize to check other SAA family members (Saa1 & Saa2) who are known to

have antibacterial properties and might have a contribution towards fungal infection clearance at that time point.

The initial investigation involves expression at early time points with SAA genes (such as *Saa1* & *Saa2*) which were found to be lower on day 2 post-infection in both Saa3 WT-OPC and Saa3-/- (KO) mice. However, on day 3 post-infection the expression was significantly reduced in *Saa3*$^{-/-}$ (KO) mice, implying that SAA genes (*Saa1 & Saa2*) were not compensating for the lack of Saa3 in *Saa3*$^{-/-}$ (KO) mice towards fungal clearance (Fig 3.2 A & B).

To further confirm these observed differences, we intended to check some antifungal genes downstream that are known to have antifungal immunity during OPC. Therefore, the gene expression pattern of *Il17, Il6, TGFβ, Tnfa, Il1β*, and AMPs (*Defb3* and *S100A9*) were studied to determine the antifungal effect in *Saa3*$^{-/-}$ (KO) mice during OPC. Initially, the expression of *Il17a* was found to be impaired in *Saa3*$^{-/-}$ (KO) OPC on days 2 & 3 after infection (Fig 3.3A), consistent with its high fungal burden at that time point. However, increased *Il17a* expression in *Saa3*$^{-/-}$ (KO) OPC at a later time point (day 4), after low expression at days 2&3, compared with *Saa3*$^{+/+}$ WT-OPC could show the activity of antifungal immune response.

Additionally, IL-17 expression is mostly triggered by *Il1β, Il6*, and transforming factor-beta (*TGFβ*) where *Il1β* is required for the development of CD4+ T cells into IL-17 generating Th17 cells and IL-17 is required for antifungal host defense (Liang et al., 2006). As a result, a deficiency in any one of them might reduce antifungal immunity by affecting IL-17 production. Similarly, the expression of *Il1β* also remained low at early time points, however, was significantly higher on day 4 in *Saa3*$^{-/-}$ (KO) OPC mice compared to sham

as controls (Fig 3.3 B). Moreover, constant reduction of *Il6* expression in *Saa3*$^{-/-}$ (KO) OPC mice till day 3, also supports their role in the reduction of antifungal immunity (Fig 3.3 C). Similarly, *TGFβ* was significantly reduced initially in *Saa3*$^{-/-}$ (KO) OPC mice (Fig 3.3 D) consistent with its role in antifungal response by producing less amount of Th17 cells producing IL-17.

Also, we analyzed the expression of *Il1a* as they are known to be released by oral epithelial cells and help in modulating the mucosal inflammatory response in respect to *Candida albicans.* On days 2&3 post-infection the expression of *Il1a* was increased in *Saa3*$^{+/+}$ (WT) OPC whereas in *Saa3*$^{-/-}$ (KO) OPC it was constantly reduced till day 3 post-infection (Fig 3.3E). However, on day 4 post-infection, the expression of *Il1a* in *Saa3*$^{-/-}$ (KO) OPC increased significantly in comparison to Sham (Fig 3.3E), suggesting its role in host defense through regulating neutrophil response at later phase of infection. Similarly, *Tnfa* which is an important component of the immune system is known to have a defensive effect against *Candida albicans* infections and is greatly generated in response to fungal infection. This further increases the development of chemokines, with the recruitment of polymorphonuclear leukocytes during fungal infection (Filler et al., 2005). Our results also showed, increased expression of *Tnfa* at day 4 post-infection in both *Saa3*$^{+/+}$ (WT) OPC and *Saa3*$^{-/-}$ (KO) OPC (Fig 3.3F) indicates the importance of *Tnfa* in response to fungal infection at later time points. However, in this study, there was no strong correlation between *Tnfα* expression levels and immune response to OPC in *Saa3*$^{-/-}$ (KO) mice.

IL-17 is further known to control the synthesis of antimicrobial peptides such as defensins (β -defensins, BDs), calprotectin (S100A8/9), and mucins in addition to contributing to the neutrophil response. Murine *-defensin 3* has already been demonstrated

to have a role in OPC protection and is IL-17-dependent. One of the first innate reactions during mucosal fungal infections is the synthesis of BD3 and other AMPs (Diamond et al., 2009). Due to their antibacterial properties and immunomodulatory role, defensins and other AMPs have attracted more interest (Ganz, 2003) (Diamond et al., 2009). Moreover, our results showed an induction of *Defb3* in *Saa3*$^{-/-}$ (KO) OPC mice at days 3 & 4 post-infection indicating its role towards fungal clearance during OPC (Fig 3.3G). However, the expression of *S100A9* was constantly reduced in *Saa3*$^{-/-}$ (KO) OPC mice (Fig 3.3H), which only suggests its role as an active immune response due to continued fungal load in *Saa3*$^{+/+}$ (WT) mice at an initial time point (day 2 post-infection) during OPC.

Further moving downstream includes chemokines CXCL1 (chemokine (C-X-C motif) ligand 1 which is a peptide that belongs to the CXC chemokine family acts as a chemoattractant produced by several immune cells, such as neutrophils, macrophages, epithelial cells, or Th17 population (Fujie et al., 2012). Moreover, during OPC, our study indicates defects in neutrophil chemokines *Cxcl1* and *Cxcl2* expression in *Saa3*$^{-/-}$ (KO) OPC mice compared with *Saa3*$^{+/+}$ (WT) OPC mice. Additionally, the expression of *Cxcl5* was comparable in both *Saa3*$^{+/+}$ (WT) OPC and *Saa3*$^{-/-}$ (KO) OPC (Fig 3.3 I, J & K). Moreover, our data were consistent with the defects in neutrophil recruitment observed in *Saa3*$^{-/-}$ (KO) OPC in comparison to *Saa3*$^{+/+}$ (WT) OPC mice (Fig 3.4 A&B). It was further supported by our flow study, which revealed that *Saa3*$^{-/-}$ (KO) OPC mice had a lower percentage of CD45, TCRβ, and GR1 cell population than *Saa3*$^{+/+}$ (WT) OPC (Fig 3.5 A, B & C). Thus, it indicates that SAA3 has a role in fungal infection with a defect in the immune cell population including neutrophils in *Saa3*$^{-/-}$ (KO) mice. This further supports that the clearance of fungal infection is solely not dependent on the neutrophil-related

pathway, like other WT mice which follow neutrophil independent mechanisms towards immunity to OPC in oral epithelial cells (Altmeier et al., 2016). Thus, the overall study suggests that SAA3 as a downstream effector could exert its antifungal properties with the help of antifungal genes and antimicrobial peptides (BD3) but at later time points, that may be involved in protection against OPC.

The findings of this thesis suggest potential areas for further research. A small area of this thesis was conducted in presence of cortisone-acetate in WT infected mice (Fig 4). Primarily, corticosteroids are known to suppress the immune system by sequestering CD4+ T-lymphocytes in the reticuloendothelial system by reducing cytokine production (Coutinho & Chapman, 2011). Surprisingly, in the presence of cortisone acetate undergoing OPC, the expression of the *Saa3* gene in cortisone-acetate WT infected mice was found to be elevated at (day 5 post-infection), indicating that there may be any inflammatory response in terms of tissue inflammation at that time point (Fig 3.2 C, D & E). However, there were insufficient data to establish the expression of *Saa3* in respect to only corticosteroids. Moreover, corticosteroids are considered as the main glucocorticoids to be involved in APR (Amrani et al., 1986) which boosts the production of the APP hormone, suggesting having an independent effect of cytokines (Moshage, 1997). The mechanism by which proinflammatory cytokines stimulate hepatic APP synthesis has been extensively studied previously. Following IL-1 interaction to the IL-6 receptor, APPs are known to get induced, followed by the phosphorylation and degradation of inhibitor kappa B (IKB). This led to further release of NF-kB and subsequent activation of the acute-phase gene in the nucleus (Curtis & Butler, 1980) (Jain et al., 2011). Therefore, in this study, the

expression of *Saa3* at this condition appears to be bad and indicates more susceptibility of *Saa3*$^{-/-}$ (KO) mice during immunosuppression.

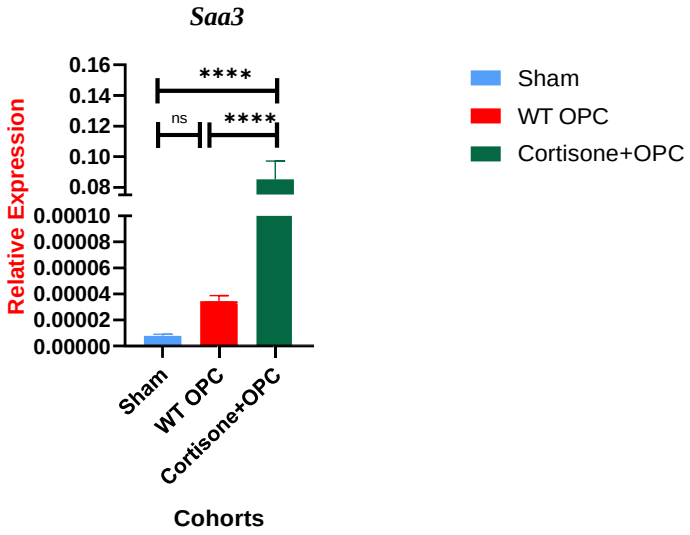


Figure 4: *Saa3* expression in cortisone-acetate WT infected mice in compared to sham mice. RNA extracted from tongue tissue harvested from cortisone-treated *C. albicans* infected mice and untreated control (Sham) was used for gene expression studies. Statistical analysis was performed using ANOVA, ****P<0.0001, ns= statistically not significant at (P< 0.05) (qPCR n=3-5) where 'n' denotes total number of mice in each group used in this experiment.

Most of the data has been found in respect to WT mice undergoing immunosuppression. But the most notable will be an investigation of these *SAA* genes in *Saa3*$^{-/-}$ (KO) mice subjected to other forms of immunosuppression such as chemotherapy & radiotherapy. This study showed the function of SAA3 with respect to fungal susceptibility. But, to understand the role of SAA3 during fungal infection, we need to study what is going on, in the background of each immunosuppression alone and in combination with OPC which is yet to be studied. Also, we need to understand the potent fungal regulation of SAA3, which is associated with the antifungal signaling pathway. However, under normal conditions, the amount of information available might be

overwhelming. Also, a number of unanticipated situations involving breeding colonies were encountered in our research, which constrained our work for a period of time to allow for further investigation.

References

Akira, S., Uematsu, S., & Takeuchi, O. (2006). Pathogen recognition and innate immunity. *Cell, 124*(4), 783-801. https://doi.org/10.1016/j.cell.2006.02.015

Altmeier, S., Toska, A., Sparber, F., Teijeira, A., Halin, C., & LeibundGut-Landmann, S. (2016). IL-1 Coordinates the Neutrophil Response to C. albicans in the Oral Mucosa. *PLoS Pathog*, *12*(9), e1005882. https://doi.org/10.1371/journal.ppat.1005882

Amrani, D. L., Mauzy-Melitz, D., & Mosesson, M. W. (1986). Effect of hepatocyte-stimulating factor and glucocorticoids on plasma fibronectin levels. *Biochem J, 238*(2), 365-371. https://doi.org/10.1042/bj2380365

Ather, J. L., Dienz, O., Boyson, J. E., Anathy, V., Amiel, E., & Poynter, M. E. (2018). Serum Amyloid A3 is required for normal lung development and survival following influenza infection. *Sci Rep*, *8*(1), 16571. https://doi.org/10.1038/s41598-018-34901-x

Benditt, E. P., & Meek, R. L. (1989). Expression of the third member of the serum amyloid A gene family in mouse adipocytes. *J Exp Med*, *169*(5), 1841-1846. https://doi.org/10.1084/jem.169.5.1841

Bonass, W. A., High, A. S., Owen, P. J., & Devine, D. A. (1999). Expression of beta-defensin genes by human salivary glands. *Oral Microbiol Immunol*, *14*(6), 371-374. https://doi.org/10.1034/j.1399-302x.1999.140607.x

Boyce, K. J., & Andrianopoulos, A. (2015). Fungal dimorphism: the switch from hyphae to yeast is a specialized morphogenetic adaptation allowing colonization of a host. *FEMS Microbiol Rev*, *39*(6), 797-811. https://doi.org/10.1093/femsre/fuv035

Calderone, R. A., & Fonzi, W. A. (2001). Virulence factors of Candida albicans. *Trends Microbiol*, *9*(7), 327-335. https://doi.org/10.1016/s0966-842x(01)02094-7

Cannon, R. D., & Chaffin, W. L. (1999). Oral colonization by Candida albicans. *Crit Rev Oral Biol Med*, *10*(3), 359-383. https://doi.org/10.1177/10454411990100030701

Chait, A., den Hartigh, L. J., Wang, S., Goodspeed, L., Babenko, I., Altemeier, W. A., & Vaisar, T. (2020). Presence of serum amyloid A3 in mouse plasma is dependent on the nature and extent of the inflammatory stimulus. *Sci Rep*, *10*(1), 10397. https://doi.org/10.1038/s41598-020-66898-7

Cheng, S. C., Joosten, L. A., Kullberg, B. J., & Netea, M. G. (2012). Interplay between Candida albicans and the mammalian innate host defense. *Infect Immun*, *80*(4), 1304-1313. https://doi.org/10.1128/IAI.06146-11

Chiba, T., Han, C. Y., Vaisar, T., Shimokado, K., Kargi, A., Chen, M. H., Wang, S., McDonald, T. O., O'Brien, K. D., Heinecke, J. W., & Chait, A. (2009). Serum amyloid A3 does not contribute to circulating SAA levels. *J Lipid Res*, *50*(7), 1353-1362. https://doi.org/10.1194/jlr.M900089-JLR200

Conti, H. R., Bruno, V. M., Childs, E. E., Daugherty, S., Hunter, J. P., Mengesha, B. G., Saevig, D. L., Hendricks, M. R., Coleman, B. M., Brane, L., Solis, N., Cruz, J. A., Verma, A. H., Garg, A. V., Hise, A. G., Richardson, J. P., Naglik, J. R., Filler, S. G., Kolls, J. K., . . . Gaffen, S. L. (2016). IL-17 Receptor Signaling in Oral Epithelial Cells Is Critical for Protection against Oropharyngeal Candidiasis. *Cell Host Microbe*, *20*(5), 606-617. https://doi.org/10.1016/j.chom.2016.10.001

Conti, H. R., & Gaffen, S. L. (2015). IL-17-Mediated Immunity to the Opportunistic Fungal Pathogen Candida albicans. *J Immunol*, *195*(3), 780-788. https://doi.org/10.4049/jimmunol.1500909

Conti, H. R., Shen, F., Nayyar, N., Stocum, E., Sun, J. N., Lindemann, M. J., Ho, A. W., Hai, J. H., Yu, J. J., Jung, J. W., Filler, S. G., Masso-Welch, P., Edgerton, M., & Gaffen, S. L. (2009). Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. *J Exp Med*, *206*(2), 299-311. https://doi.org/10.1084/jem.20081463

Couderc, E., Morel, F., Levillain, P., Buffiere-Morgado, A., Camus, M., Paquier, C., Bodet, C., Jegou, J. F., Pohin, M., Favot, L., Garcia, M., Huguier, V., McHeik, J., Lacombe, C., Yssel, H., Guillet, G., Bernard, F. X., & Lecron, J. C. (2017). Interleukin-17A-induced production of acute serum amyloid A by keratinocytes

contributes to psoriasis pathogenesis. *PLoS One*, *12*(7), e0181486. https://doi.org/10.1371/journal.pone.0181486

Coutinho, A. E., & Chapman, K. E. (2011). The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. *Mol Cell Endocrinol*, *335*(1), 2-13. https://doi.org/10.1016/j.mce.2010.04.005

Cray, C., Zaias, J., & Altman, N. H. (2009). Acute phase response in animals: a review. *Comp Med*, *59*(6), 517-526. https://www.ncbi.nlm.nih.gov/pubmed/20034426

Cua, D. J., & Tato, C. M. (2010). Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol*, *10*(7), 479-489. https://doi.org/10.1038/nri2800

Curtis, M. J., & Butler, E. J. (1980). Response of caeruloplasmin to Escherichia coli endotoxins and adrenal hormones in the domestic fowl. *Res Vet Sci*, *28*(2), 217-222. https://www.ncbi.nlm.nih.gov/pubmed/6251525

De Bernardis, F., Arancia, S., Morelli, L., Hube, B., Sanglard, D., Schafer, W., & Cassone, A. (1999). Evidence that members of the secretory aspartyl proteinase gene family, in particular SAP2, are virulence factors for Candida vaginitis. *J Infect Dis*, *179*(1), 201-208. https://doi.org/10.1086/314546

Diamond, G., Beckloff, N., Weinberg, A., & Kisich, K. O. (2009). The roles of antimicrobial peptides in innate host defense. *Curr Pharm Des*, *15*(21), 2377-2392. https://doi.org/10.2174/138161209788682325

Eckersall, P. D., Young, F. J., McComb, C., Hogarth, C. J., Safi, S., Weber, A., McDonald, T., Nolan, A. M., & Fitzpatrick, J. L. (2001). Acute phase proteins in serum and milk from dairy cows with clinical mastitis. *Vet Rec*, *148*(2), 35-41. https://doi.org/10.1136/vr.148.2.35

Fan, Y., Zhang, G., Vong, C. T., & Ye, R. D. (2020). Serum amyloid A3 confers protection against acute lung injury in Pseudomonas aeruginosa-infected mice. *Am J Physiol Lung Cell Mol Physiol*, *318*(2), L314-L322. https://doi.org/10.1152/ajplung.00309.2019

Filler, S. G., Yeaman, M. R., & Sheppard, D. C. (2005). Tumor necrosis factor inhibition and invasive fungal infections. *Clin Infect Dis*, *41 Suppl 3*, S208-212. https://doi.org/10.1086/430000

Fujie, H., Niu, K., Ohba, M., Tomioka, Y., Kitazawa, H., Nagashima, K., Ohrui, T., & Numasaki, M. (2012). A distinct regulatory role of Th17 cytokines IL-17A and IL-17F in chemokine secretion from lung microvascular endothelial cells. *Inflammation*, *35*(3), 1119-1131. https://doi.org/10.1007/s10753-011-9419-0

Gaffen, S. L. (2009). Structure and signalling in the IL-17 receptor family. *Nat Rev Immunol*, *9*(8), 556-567. https://doi.org/10.1038/nri2586

Ganz, T. (2003). Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol*, *3*(9), 710-720. https://doi.org/10.1038/nri1180

Ge, Y., Huang, M., & Yao, Y. M. (2020). Biology of Interleukin-17 and Its Pathophysiological Significance in Sepsis. *Front Immunol*, *11*, 1558. https://doi.org/10.3389/fimmu.2020.01558

Gow, N. A., van de Veerdonk, F. L., Brown, A. J., & Netea, M. G. (2011). Candida albicans morphogenesis and host defence: discriminating invasion from colonization. *Nat Rev Microbiol*, *10*(2), 112-122. https://doi.org/10.1038/nrmicro2711

Hall, R. A. (2015). Dressed to impress: impact of environmental adaptation on the Candida albicans cell wall. *Mol Microbiol*, *97*(1), 7-17. https://doi.org/10.1111/mmi.13020

Hardison, S. E., & Brown, G. D. (2012). C-type lectin receptors orchestrate antifungal immunity. *Nat Immunol*, *13*(9), 817-822. https://doi.org/10.1038/ni.2369

Hari-Dass, R., Shah, C., Meyer, D. J., & Raynes, J. G. (2005). Serum amyloid A protein binds to outer membrane protein A of gram-negative bacteria. *J Biol Chem*, *280*(19), 18562-18567. https://doi.org/10.1074/jbc.M500490200

Hernandez-Santos, N., & Gaffen, S. L. (2012). Th17 cells in immunity to Candida albicans. *Cell Host Microbe*, *11*(5), 425-435. https://doi.org/10.1016/j.chom.2012.04.008

Hoyer, L. L., Green, C. B., Oh, S. H., & Zhao, X. (2008). Discovering the secrets of the Candida albicans agglutinin-like sequence (ALS) gene family--a sticky pursuit. *Med Mycol*, *46*(1), 1-15. https://doi.org/10.1080/13693780701435317

Jain, S., Gautam, V., & Naseem, S. (2011). Acute-phase proteins: As diagnostic tool. *J Pharm Bioallied Sci*, *3*(1), 118-127. https://doi.org/10.4103/0975-7406.76489

Korn, T., Bettelli, E., Oukka, M., & Kuchroo, V. K. (2009). IL-17 and Th17 Cells. *Annu Rev Immunol*, *27*, 485-517. https://doi.org/10.1146/annurev.immunol.021908.132710

Kuret, T., Lakota, K., Mali, P., Cucnik, S., Praprotnik, S., Tomsic, M., & Sodin-Semrl, S. (2018). Naturally occurring antibodies against serum amyloid A reduce IL-6 release from peripheral blood mononuclear cells. *PLoS One*, *13*(4), e0195346. https://doi.org/10.1371/journal.pone.0195346

Lachmann, H. J., Goodman, H. J., Gilbertson, J. A., Gallimore, J. R., Sabin, C. A., Gillmore, J. D., & Hawkins, P. N. (2007). Natural history and outcome in systemic AA amyloidosis. *N Engl J Med*, *356*(23), 2361-2371. https://doi.org/10.1056/NEJMoa070265

Larson, M. A., Wei, S. H., Weber, A., Mack, D. R., & McDonald, T. L. (2003). Human serum amyloid A3 peptide enhances intestinal MUC3 expression and inhibits EPEC adherence. *Biochem Biophys Res Commun*, *300*(2), 531-540. https://doi.org/10.1016/s0006-291x(02)02901-7

Lee, H. Y., Kim, S. D., Shim, J. W., Lee, S. Y., Yun, J., & Bae, Y. S. (2009). LL-37 inhibits serum amyloid A-induced IL-8 production in human neutrophils. *Exp Mol Med*, *41*(5), 325-333. https://doi.org/10.3858/emm.2009.41.5.036

Liang, S. C., Tan, X. Y., Luxenberg, D. P., Karim, R., Dunussi-Joannopoulos, K., Collins, M., & Fouser, L. A. (2006). Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med*, *203*(10), 2271-2279. https://doi.org/10.1084/jem.20061308

Marhaug, G., Hackett, B., & Dowton, S. B. (1997). Serum amyloid A gene expression in rabbit, mink and mouse. *Clin Exp Immunol*, *107*(2), 425-434. https://doi.org/10.1111/j.1365-2249.1997.287-c31178.x

Martin-Orozco, N., Muranski, P., Chung, Y., Yang, X. O., Yamazaki, T., Lu, S., Hwu, P., Restifo, N. P., Overwijk, W. W., & Dong, C. (2009). T helper 17 cells promote cytotoxic T cell activation in tumor immunity. *Immunity*, *31*(5), 787-798. https://doi.org/10.1016/j.immuni.2009.09.014

Martin, R., Moran, G. P., Jacobsen, I. D., Heyken, A., Domey, J., Sullivan, D. J., Kurzai, O., & Hube, B. (2011). The Candida albicans-specific gene EED1 encodes a key regulator of hyphal extension. *PLoS One*, *6*(4), e18394. https://doi.org/10.1371/journal.pone.0018394

Mayer, F. L., Wilson, D., & Hube, B. (2013). Candida albicans pathogenicity mechanisms. *Virulence*, *4*(2), 119-128. https://doi.org/10.4161/viru.22913

Mengesha, B. G., & Conti, H. R. (2017). The Role of IL-17 in Protection against Mucosal Candida Infections. *J Fungi (Basel)*, *3*(4). https://doi.org/10.3390/jof3040052

Monin, L., & Gaffen, S. L. (2018). Interleukin 17 Family Cytokines: Signaling Mechanisms, Biological Activities, and Therapeutic Implications. *Cold Spring Harb Perspect Biol*, *10*(4). https://doi.org/10.1101/cshperspect.a028522

Moshage, H. (1997). Cytokines and the hepatic acute phase response. *J Pathol*, *181*(3), 257-266. https://doi.org/10.1002/(SICI)1096-9896(199703)181:3<257::AID-PATH756>3.0.CO;2-U

Naglik, J. R., Challacombe, S. J., & Hube, B. (2003). Candida albicans secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol Mol Biol Rev*, *67*(3), 400-428, table of contents. https://doi.org/10.1128/MMBR.67.3.400-428.2003

Naglik, J. R., & Moyes, D. (2011). Epithelial cell innate response to Candida albicans. *Adv Dent Res*, *23*(1), 50-55. https://doi.org/10.1177/0022034511399285

Netea, M. G., Gow, N. A., Munro, C. A., Bates, S., Collins, C., Ferwerda, G., Hobson, R. P., Bertram, G., Hughes, H. B., Jansen, T., Jacobs, L., Buurman, E. T., Gijzen, K.,

Williams, D. L., Torensma, R., McKinnon, A., MacCallum, D. M., Odds, F. C., Van der Meer, J. W., . . . Kullberg, B. J. (2006). Immune sensing of Candida albicans requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. *J Clin Invest*, *116*(6), 1642-1650. https://doi.org/10.1172/JCI27114

Netea, M. G., Joosten, L. A., van der Meer, J. W., Kullberg, B. J., & van de Veerdonk, F. L. (2015). Immune defence against Candida fungal infections. *Nat Rev Immunol*, *15*(10), 630-642. https://doi.org/10.1038/nri3897

O'Reilly, E. L., & Eckersall, P. D. (2014). Acute phase proteins: a review of their function, behaviour and measurement in chickens. *World's Poultry Science Journal*, *70*(1), 27-44. https://doi.org/10.1017/S0043933914000038

Pankhurst, C. L. (2013). Candidiasis (oropharyngeal). *BMJ Clin Evid*, *2013*, 1304. https://www.ncbi.nlm.nih.gov/pubmed/24209593

Patin, E. C., Thompson, A., & Orr, S. J. (2019). Pattern recognition receptors in fungal immunity. *Semin Cell Dev Biol*, *89*, 24-33. https://doi.org/10.1016/j.semcdb.2018.03.003

Pfaller, M. A., & Diekema, D. J. (2007). Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev*, *20*(1), 133-163. https://doi.org/10.1128/CMR.00029-06

Rabeneck, L., Crane, M. M., Risser, J. M., Lacke, C. E., & Wray, N. P. (1993). A simple clinical staging system that predicts progression to AIDS using CD4 count, oral thrush, and night sweats. *J Gen Intern Med*, *8*(1), 5-9. https://doi.org/10.1007/BF02600284

Santiago-Cardona, P. G., Berrios, C. A., Ramirez, F., & Garcia-Arraras, J. E. (2003). Lipopolysaccharides induce intestinal serum amyloid A expression in the sea cucumber Holothuria glaberrima. *Dev Comp Immunol*, *27*(2), 105-110. https://doi.org/10.1016/s0145-305x(02)00068-x

Saul-McBeth, J., Dillon, J., Lee, A., Launder, D., Kratch, J. M., Abutaha, E., Williamson, A. A., Schroering, A. G., Michalski, G., Biswas, P., Conti, S. R., 3rd, Shetty, A. C., McCracken, C., Bruno, V. M., Parsai, E. I., & Conti, H. R. (2021). Tissue Damage

in Radiation-Induced Oral Mucositis Is Mitigated by IL-17 Receptor Signaling. *Front Immunol*, *12*, 687627. https://doi.org/10.3389/fimmu.2021.687627

Schrodl, W., Buchler, R., Wendler, S., Reinhold, P., Muckova, P., Reindl, J., & Rhode, H. (2016). Acute phase proteins as promising biomarkers: Perspectives and limitations for human and veterinary medicine. *Proteomics Clin Appl*, *10*(11), 1077-1092. https://doi.org/10.1002/prca.201600028

Shah, C., Hari-Dass, R., & Raynes, J. G. (2006). Serum amyloid A is an innate immune opsonin for Gram-negative bacteria. *Blood*, *108*(5), 1751-1757. https://doi.org/10.1182/blood-2005-11-011932

Singh, A. K., Gaur, P., Shukla, N. K., & Das, S. N. (2015). Differential dendritic cell-mediated activation and functions of invariant NKT-cell subsets in oral cancer. *Oral Dis*, *21*(1), e105-113. https://doi.org/10.1111/odi.12238

Sproston, N. R., & Ashworth, J. J. (2018). Role of C-Reactive Protein at Sites of Inflammation and Infection. *Front Immunol*, *9*, 754. https://doi.org/10.3389/fimmu.2018.00754

Staab, J. F., Datta, K., & Rhee, P. (2013). Niche-specific requirement for hyphal wall protein 1 in virulence of Candida albicans. *PLoS One*, *8*(11), e80842. https://doi.org/10.1371/journal.pone.0080842

Steel, D. M., & Whitehead, A. S. (1994). The major acute phase reactants: C-reactive protein, serum amyloid P component and serum amyloid A protein. *Immunol Today*, *15*(2), 81-88. https://doi.org/10.1016/0167-5699(94)90138-4

Sullivan, D., & Coleman, D. (1998). Candida dubliniensis: characteristics and identification. *J Clin Microbiol*, *36*(2), 329-334. https://doi.org/10.1128/JCM.36.2.329-334.1998

Tannock, L. R., De Beer, M. C., Ji, A., Shridas, P., Noffsinger, V. P., den Hartigh, L., Chait, A., De Beer, F. C., & Webb, N. R. (2018). Serum amyloid A3 is a high density lipoprotein-associated acute-phase protein. *J Lipid Res*, *59*(2), 339-347. https://doi.org/10.1194/jlr.M080887

Toy, D., Kugler, D., Wolfson, M., Vanden Bos, T., Gurgel, J., Derry, J., Tocker, J., & Peschon, J. (2006). Cutting edge: interleukin 17 signals through a heteromeric receptor complex. *J Immunol*, *177*(1), 36-39. https://doi.org/10.4049/jimmunol.177.1.36

Tsuchimori, N., Sharkey, L. L., Fonzi, W. A., French, S. W., Edwards, J. E., Jr., & Filler, S. G. (2000). Reduced virulence of HWP1-deficient mutants of Candida albicans and their interactions with host cells. *Infect Immun*, *68*(4), 1997-2002. https://doi.org/10.1128/IAI.68.4.1997-2002.2000

Uhlar, C. M., & Whitehead, A. S. (1999). Serum amyloid A, the major vertebrate acute-phase reactant. *Eur J Biochem*, *265*(2), 501-523. https://doi.org/10.1046/j.1432-1327.1999.00657.x

Vaure, C., & Liu, Y. (2014). A comparative review of toll-like receptor 4 expression and functionality in different animal species. *Front Immunol*, *5*, 316. https://doi.org/10.3389/fimmu.2014.00316

Vila, T., Sultan, A. S., Montelongo-Jauregui, D., & Jabra-Rizk, M. A. (2020). Oral Candidiasis: A Disease of Opportunity. *J Fungi (Basel)*, *6*(1). https://doi.org/10.3390/jof6010015

Weindl, G., Wagener, J., & Schaller, M. (2011). Interaction of the mucosal barrier with accessory immune cells during fungal infection. *Int J Med Microbiol*, *301*(5), 431-435. https://doi.org/10.1016/j.ijmm.2011.04.011

Zhang, Q. Y., Yan, Z. B., Meng, Y. M., Hong, X. Y., Shao, G., Ma, J. J., Cheng, X. R., Liu, J., Kang, J., & Fu, C. Y. (2021). Antimicrobial peptides: mechanism of action, activity and clinical potential. *Mil Med Res*, *8*(1), 48. https://doi.org/10.1186/s40779-021-00343-2

Zheng, H., Li, H., Zhang, J., Fan, H., Jia, L., Ma, W., Ma, S., Wang, S., You, H., Yin, Z., & Li, X. (2020). Serum amyloid A exhibits pH dependent antibacterial action and contributes to host defense against Staphylococcus aureus cutaneous infection. *J Biol Chem*, *295*(9), 2570-2581. https://doi.org/10.1074/jbc.RA119.010626