Induction of the Epithelial Polarizing Cytokine Interleukin-33 by the Fungus *Cryptococcus neoformans* in Genetically Susceptible Mice

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*Table 1:* The protective and pathological roles of the epithelial polarizing cytokines TSLP, IL-25 and IL-33 in disease .................................................................................. 30
LIST OF ABBREVIATIONS

AAM – Alternative Activation of Macrophages
AHR – Airway Hyper-Responsiveness
AIDS – Acquired Immunodeficiency Syndrome
AM – Alveolar Macrophage
APC – Antigen Presenting Cell
BAL – Bronchoalveolar Lavage
CAM – Classical Activation of Macrophages
CFU – Colony-Forming Units
CNS – Central Nervous System
EAE – Experimental Autoimmune Encephalomyelitis
ELISA – Enzyme-Linked Immunosorbent Assay
ERK – Extracellular Signal-Regulated Kinase
GXM – Glucoronoxylomannan
HEV – High Endothelial Venule
HIV – Human Immunodeficiency Virus
IFN-γ – Interferon-Gamma
Ig – Immunoglobulin
IL – Interleukin
IL-1RAP – IL-1 Receptor Accessory Protein
IRAK – IL-1R-Associated Kinase
KO - Knockout
LALN – Lung Associated Lymph Node
MOI – Multiplicity of Infection
MyD88 – Myeloid Differentiation Primary Response Gene 88
NFκB – Nuclear Factor-Kappa B
PRR – Pattern Recognition Receptor
SDA – Sabouraud Dextrose Agar
ST2 – Suppression of Tumorigenicity 2
TGF-β – Transforming Growth Factor-Beta
TH1 – T-Helper Type 1
TLR – Toll-Like Receptor
TNF-α – Tumor Necrosis Factor Alpha
Treg – T-Regulatory cells
TSLP – Thymic Stromal Lymphopoietin
ABSTRACT

With the progression of the AIDS epidemic, understanding the host response to the opportunistic fungal pathogen *Cryptococcus neoformans*, responsible for approximately 650,000 deaths in Sub-Saharan Africa each year, has become an important topic of research. Current knowledge suggests that susceptibility to cryptococcal pneumonia in both mice and humans proceeds via an allergic (TH2) pattern of lung inflammation, while resistance is attributable to a TH1 response in the lungs. The epithelial polarizing cytokines thymic stromal lymphopoietin (TSLP), interleukin-25 (IL-25) and IL-33 have been implicated in TH2 mucosal and respiratory inflammation caused by allergens and helminths; however, their roles during *C. neoformans* infection are not known. We demonstrated that *in vitro* stimulation of the mouse lung epithelial cell line (MLE-12) with both the acapsular mutant *C. neoformans* CAP64 and the highly virulent *C. neoformans* H99, resulted in dose- and time-dependent increases in both *Il25* and *Il33* mRNA expression. Correspondingly, intranasal infection of susceptible Balb/c mice with *C. neoformans* H99 showed time-dependent IL-33 mRNA and protein in the lungs. Furthermore, moderately virulent *C. neoformans* 52D induced differential *Il33* mRNA expression among susceptible and resistant strains of mice, with susceptible C57BL/6 mice developing a significant increase in lung *Il33* mRNA compared to resistant CBA mice. Finally, Balb/c mice lacking the IL-33 receptor T1/ST2 had significantly reduced lung, spleen and brain fungal burdens following intratracheal instillation of *C. neoformans*. These observations support a role for IL-33 in polarization of the host inflammatory response that facilitates progressive pulmonary *C. neoformans* infection.
Avec la prévalence de l’épidémie du SIDA, comprendre la réaction de l’hôte au champignon opportuniste pathogène Cryptococcus neoformans, responsable de 650,000 décès chaque année en Afrique sub-saharienne, est devenu un sujet de recherche profonde. Les connaissances actuelles suggèrent que la susceptibilité à la pneumonie de cryptocoque chez les souris et les humains se produit par l’intermédiaire des réponses allergiques de type Th2 d'inflammation pulmonaire, alors que la résistance est attribuable au développement d’une réponse Th1 dans les poumons. Les cytokines épithéliales polarisatrices comme thymic stromal lymphopoietin (TSLP), l’interleukine-25 (IL-25) et IL-33 sont impliqués dans l'inflammation allergique Th2 respiratoire, mais leur rôle pendant l’infection C. neoformans reste inconnu. Nous démontrons que la stimulation in vitro des cellules épithéliales de poumons de souris (MLE-12) par la souche C. neoformans sans-capsule CAP64 ou la souche C. neoformans très virulente H99, révèle une augmentation sélective de doses de l'expression d'ARNm d'IL25 et d'IL33. Corrélativement, l'infection intranasale de souris susceptible Balb/c au H99 a montré l'induction en fonction du temps de l'ARNm et de la protéine de l'IL-33 dans les poumons. Par ailleurs, l’utilisation de la souche de C. neoformans modérément virulente 52D, a démontré une expression de l’IL33 différente entre les souches de souris susceptibles et résistantes. La souche C57BL/6 a connu une augmentation significative de l'ARNm de l’IL33 dans les poumons comparée à la souche résistante CBA. Enfin, les souris Balb/c knockout qui manquaient le récepteur T1/ST2 pour l’IL-33, avaient une charge fongique réduite dans les poumons, les spleens et les cerveaux suite à l’infection par C. neoformans. Ces observations suggèrent un rôle désavantageux de l’IL-33 dans la polarisation de la réponse inflammatoire pendant l’infection C. neoformans pulmonaire.
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INTRODUCTION

Rationale and objectives

In 1969, the Surgeon General of the United States told Congress somewhat prematurely “it was time to close the book on infectious diseases (1,2).” To develop the most effective disease prevention and management strategies, it is crucial to delineate the underlying inflammatory and immune pathways that control host defense against infection. With new emerging and reemerging infectious diseases, an aging population, and increased numbers of immune compromised individuals (2), it is essential that we find new therapeutic avenues to combat infectious diseases.

Along with a reemergence of certain infectious diseases, an increase in allergic diseases is occurring in the westernized world. Currently, over 300 million people worldwide suffer from asthma or allergic diseases, and this number is not diminishing (3). Though infectious and allergic diseases were once considered to represent opposing or divergent processes, it is now known that the same mechanisms that govern the development of asthma are responsible for host protective immunity to helminths and nematodes (4). This suggests that allergic responses could play a role during infectious diseases, a concept that warrants further exploration. Recent observations suggest an association exists between allergic inflammation and poor outcomes during *Leishmania major* (5) and *Cryptococcus neoformans* (6) infections in both humans and animals.
Our group and others are specifically interested in how allergic responses drive susceptibility to *C. neoformans* infection. Infections with pathogenic fungi are on the rise in an era where immunosuppression associated with organ transplantation is commonplace and where new and growing populations, such as patients with Acquired Immune Deficiency Syndrome (AIDS) are predisposed to increased rates of severe infection (7). *Cryptococcus* is the 4\textsuperscript{th} most common fatal infectious disease in sub-Saharan Africa and it is beginning to emerge as a pathogen in immune competent individuals as well (8). Inhalation of *C. neoformans* propagules leads to lung infection, pneumonia, and disseminated disease involving the central nervous system (CNS) that is uniformly fatal if left untreated. Although asthma and *C. neoformans* infection have not been formally linked, recent studies in asthmatic children showed high *C. neoformans* chitinase protein in their airways (9). With current knowledge suggesting that cryptococcal infection progresses via a T\textsubscript{H}2-type immunopathology in both mice and humans (7), it may be possible to utilize insights from the study of allergic diseases and asthma to elucidate novel pathways responsible for host susceptibility to *C. neoformans*.

**The fungus Cryptococcus**

The kingdom Fungi encompasses over 1.5 million eukaryotic members that have many biological processes in common with plants and animals (10). Fungi come in many varieties including yeasts, molds and mushrooms, and can be found in every continent on the planet (10). We are constantly in contact with fungi as they make up approximately 10\% of airborne particulate matter (11).
*Cryptococcus* is a basidiomycetous yeast-like fungus that is ubiquitous in the environment. Following isolation from the environment as a budding yeast, *Cryptococcus* can undergo mating and monokaryotic fruiting to transition into a filamentous form (12). The filamentous form allows the fungus to scavenge for nutrients and permits the production of spores (13). These spores, if inhaled, can cause respiratory disease with subsequent CNS dissemination in both humans and animals (8,14,15). *Cryptococcus* is found mainly in soil, avian guano, rotting vegetables and decomposing wood (14). There are three varieties and four unique serotypes of *Cryptococcus* (*Figure 1*). *Cryptococcus* var. *grubii* (serotype A), responsible for 95% of clinical infections, and *Cryptococcus* var. *neoformans* (serotype D), both cause disease in immunocompromised hosts and are ubiquitously present in the environment (13). A related species, found in tropical and subtropical climate zones, known as *Cryptococcus gattii* (serotypes B and C) is responsible for approximately 1% of clinical infections in immunocompetent patients (15).

To be pathogenic, *Cryptococcus* has had to evolve unique evasion strategies, including virulence factors, to survive within the human body. It is believed that these virulence factors may have originally evolved as defense mechanisms against environmental predators (16). Usually identified by its large polysaccharide capsule and melanin pigmented cell wall, *Cryptococcus* is able to use these unique virulence factors, combined with its ability to grow at human body temperature (17) to subvert host defenses (13). The carbohydrate rich outer capsule, composed primarily of glucoronoxylomannan (GXM), can help *Cryptococcus* avoid desiccation, can prevent internalization by host phagocytes
(18), can alter the hosts chemokine and cytokine profile (19), and along with melanin can protect *Cryptococcus* from damaging ultraviolet radiation and host reactive oxygen species (20,21). Other unique evasion and infection strategies include the use of extracellular proteases such as urease to help it penetrate the blood brain barrier (22). It is ultimately these virulence factors that have made *Cryptococcus* such a successful human pathogen.

**Cryptococcal disease and epidemiology**

*Cryptococcus neoformans* is a ubiquitous environmental fungal pathogen that became a significant cause of human disease since the advent of the human immunodeficiency virus (HIV) in 1981 (23). With the discovery of highly active antiretroviral therapy (HAART) to treat HIV, *C. neoformans* infections have decreased in North America (24,25); yet, in spite of this, *C. neoformans* still remains one of the leading causes of death in the Acquired Immune Deficiency Syndrome (AIDS) population of Sub-Saharan Africa (26). Cryptococcosis is responsible for up to 30% of deaths in AIDS patients and is believed to be one of the leading causes of mortality in transplant recipients (27). Infection by *Cryptococcus* occurs by inhalation of infectious yeast propagules from the environment (23) (*Figure 2*). Although extremely rare, cases of human-human transmission have been reported and mouse studies have found shedding of cryptococcal cells into the bedding of their cages (28,29). In a sample from New York City, the majority of children ≥2 years of age had serological evidence of antibody reactivity to cryptococcal protein, with no overt history of disease (30).
This suggests that immunocompetent hosts generally develop self-limited or latent cryptococcal disease that may become reactivated upon immune suppression (23).

Inhalation of *C. neoformans* by healthy individuals leads to mild, latent or asymptomatic disease, while immune compromised individuals may develop severe pneumonia and disseminated disease leading to meningoencephalitis (24). Pulmonary infections are generally asymptomatic; however, patients may present with cough, pleuritic chest pain, fever, dyspnea, weight loss, malaise and in severe cases pneumonia and respiratory distress syndrome (31). Symptoms of meningoencephalitis include headache, fever, visual problems and an altered mental state that, and if left untreated, will invariably lead to death (32).

Clinical detection relies on the use of chest x-rays to detect pulmonary nodules, enzyme-linked immunosorbent assays (ELISA) to detect cryptococcal proteins, cultured sputum and bronchoalveolar lavage (BAL) samples to identify the *Cryptococcus* strain, and cerebral spinal fluid samples to detect dissemination to the central nervous system (33).

Current treatment options for fungal infections remain limited and, as fungi are Eukaryotes, they present unique challenges for drug development (34,35). Currently, systemic antifungal combination therapy with Amphotericin B, Flucytosine and Fluconazole is the standard treatment for cryptococcal infection (36). Even with expeditious antifungal treatment, mortality still remains unacceptably high (37); for example, the 3-month mortality rate for HIV patients with cryptococcal meningoencephalitis is approximately 20% (38,39).

A recent outbreak of a related species, *Cryptococcus gattii*, in British Columbia, Canada and in the Pacific Northwest of the United States, has caused
disease in hundreds of apparently immune competent humans and animals (15). This epidemic is remarkable since fungi rarely cause mortality in the healthy human population. Other than some common superficial infections such as athlete’s foot and vaginitis, fungi are generally non-pathogenic to humans (13). Thus, in order to stop the potential spread of \textit{C. gattii} in immunocompetent individuals it is imperative that we find new novel treatments for this emerging fungal pathogen.

\textbf{Animal models of Cryptococcosis}

\textit{Cryptococcus} has a broad host range that allows for the utilization of animal models to study cryptococcal pathogenesis, virulence, immunology, diagnosis, and therapy (40). Animal models of infection attempt to recapitulate what is observed during human disease while allowing for control of variables such as dose, route of infection, and strain of microorganism. These experimental systems can provide invaluable information on the host-pathogen interactions that occur \textit{in vivo}, yet each has certain limitations (25). For example, disadvantages of large animal models include the associated costs, animal infections are generally more acute than clinical human infection, organ tropisms of pathogens may differ between humans and animals, and drug metabolism varies greatly from species to species (40).

The nematode \textit{Caenorhabditis elegans} is one model of \textit{C. neoformans} infection that is highly susceptible to \textit{C. neoformans} infection. As with mammalian hosts, the absence of virulence genes such as those associated with capsule production allow \textit{C. elegans} to clear cryptococcal infection (41). This data
suggests that *C. neoformans* has evolved these virulence factors to survive natural predators in its environment, and as an unintended consequence has become pathogenic for mammalian hosts (42).

Mammalian hosts that can be infected with *C. neoformans* include humans, dogs, cats, rabbits, mice, horses, cows and primates among others (25). Most often, small mammals such as guinea pigs, rabbits, rats and mice are used for their ease of breeding and low costs of maintenance. As dissemination of *C. neoformans* to the CNS is what ultimately causes death in humans, many animal models attempt to reproduce the same pathologic process (43). Intraperitoneal infection is least similar to clinical disease, although it has been shown to result in dissemination to the CNS. Direct CNS involvement can be studied by intracerebral inoculation. Rabbits serve as excellent models of cryptococcal meningitis, however, rabbits are naturally resistant to cryptococcosis and therefore require immunosuppressive drugs prior to infection (44). Ultimately, natural cryptococcal infection occurs via inhalation of *C. neoformans*, therefore both intranasal and intratracheal infections seem to best replicate human clinical disease (25).

With the recent high density mapping of all single nucleotide polymorphisms of the laboratory mouse, mice have become an invaluable tool for both immunologic and genetic studies of host susceptibility (45). The wide availability of mice with spontaneous immunological defects and engineered deletion of specific genes, makes them particularly valuable tools for the study of the host immune response (46). It is now even possible to insert human genes into mice, creating transgenic humanized mice that can be utilized to study the
functioning of human genes \textit{in vivo} during infection (47). Unlike human infection, mice do not require immunosuppression and are quite susceptible to intraperitoneal, intravenous, intranasal and intratracheal infection with \textit{C. neoformans}. Different inbred strains of mice show a wide spectrum of natural susceptibility to cryptococcal disease (25,48). A strain survey, performed in our lab, clearly shows that C57BL/6 mice are highly susceptible to pulmonary growth of \textit{C. neoformans} cells, whereas the strains CBA and SJL are relatively resistant (49) (Figure 3). This type of information has encouraged many investigators to utilize mice as an excellent model host for \textit{C. neoformans} infection.

\textbf{The immune response to \textit{C. neoformans} infection}

Infectious spore propagules inhaled by the host establish infection in the distal airways and lungs (50). There they can replicate and survive either extracellularly, where they are perpetually in contact with the respiratory epithelium, or intracellularly where they are taken up by phagocytes such as alveolar macrophages (AMs) (51), dendritic cells (52) and neutrophils (53). At the host cell surface, the cryptococcal capsular polysaccharide GXM is recognized by a variety of pattern recognition receptors (PRR) involved in innate immunity including Toll-like receptor 2 (TLR2), TLR4 and CD14 (54). Interestingly, mice devoid of TLR2, CD14 or the intracellular signaling adaptor Myeloid differentiation primary response gene 88 (MyD88) used by many PRRs, were susceptible to \textit{C. neoformans} infection with an increased mortality compared to wild type mice (54).
The specific role of the respiratory epithelium during *C. neoformans* infection has not been well established; however, studies in our laboratory have shown that the human bronchial epithelial cell line (BEAS-2B) secretes IL-8, a neutrophil chemoattractant, in response to cryptococcal challenge. These results suggest that the earliest contribution of the innate immune response to *C. neoformans* infection may well originate from the epithelial lining of the respiratory tract (55). The role of AMs and other phagocytes has been well established during *C. neoformans* infection. Phagocytosis and subsequent intracellular growth of *C. neoformans* can be a determinant of susceptibility. For example, AMs from rats are not permissive to *C. neoformans* growth, whereas the AMs from mice allow the growth of cryptococcal cells within their phagolysosomes (18). It is for this reason that *Cryptococcus* is considered an intracellular facultative pathogen (56). The fate of *Cryptococcus* within host macrophages can be quite varied. *Cryptococcus* can be killed, enter a latent state, can escape via lytic and non-lytic means, or it can be laterally transferred between adjacent macrophages (18). Although AMs are one of the first lines of defense against *C. neoformans*, two recent studies have shown that the absence of monocytes can indeed be beneficial to the host (57,58). This can be partially explained because a protective response to *C. neoformans* necessitates the activation of the T-helper type 1 (T<sub>H</sub>1) immune response characterized by the classical activation of macrophages (CAM) and the presence of proinflammatory cytokines interferon-γ (INF-γ) and tumor necrosis factor-α (TNF-α), in both humans and mouse models of disease (59). On the other hand, a defect in, or
absence of proinflammatory mediators will lead to an allergic Th2 type of immunity characterized by alternative activation of macrophages (AAM), production of interleukin-4 (IL-4), IL-5 and IL-13 cytokines, lung eosinophilia, and high immunoglobulin E (IgE) in the lungs (60). AAM have been demonstrated to harbor *C. neoformans* and facilitate its growth, leading to severe lung pathology and dissemination of disease in clinically relevant mouse models of cryptococcal infection (60).

Although extremely rare, *C. neoformans* infection has also been shown to occur in patients lacking neutrophils (61). In our lab it was shown that the inbred strain SJL/J, which is highly resistant to intratracheal infection with *C. neoformans* 52D, had significant neutrophilia compared to the susceptible C57BL/6 (49) The Th17 immune response defined by IL-17 secretion and neutrophil activation was also shown to be protective against *C. neoformans* infection (62). Maintenance of the unique Th17 response requires the presence of IL-23. Exogenous administration of this cytokine to mice infected with *C. neoformans* led to prolonged survival and reduced fungal burdens (63). Evidence currently suggests that neutrophils kill cryptococcal cells using a variety of oxidative and non-oxidative mechanisms including oxidative burst and defensins (64,65).

Dendritic cells (DCs), in addition to macrophages, have also been shown to play a role in the immune response to *C. neoformans* via antigen presentation. They pick up cryptococcal antigen in the lungs via a combination of their mannose and Fcγ receptors and present it to T-lymphocytes in the lung associated lymph nodes (LALN) to activate adaptive immunity (52). DCs are believed to
provide the crucial link between innate and adaptive immunity during *C. neoformans* infection, and depletion of DCs leads to a loss of T cell mediated immunity (66). Ultimately it is the instruction provided by phagocytes, airway epithelium and other innate mechanisms that determine the balance of T\(_H\)1-T\(_H\)2-T\(_H\)17 response, which is important for the clearance and survival of *C. neoformans* infection in both mice and man (8).

**Adaptive immune responses to *C. neoformans***

CD4\(^+\) T cells are indispensible to the immune response to infection. They are important for helping B cells mediate an antibody response, for recruiting and activating immune cells such as macrophages, neutrophils, eosinophils and basophils, and are important for their capacity to secrete chemokines and cytokines to direct and orchestrate the immune response to pathogens (67). CD4\(^+\) T cells emerge from the thymus as either naïve, as natural T-regulatory cells (68) or as Natural Killer T cells (69). It was discovered that depending on the pattern of signals and the nature of the antigen that they first encounter, naïve CD4\(^+\) T cells can become one of 4 distinct populations: T-helper type 1 (T\(_H\)1), T\(_H\)2, T\(_H\)17 or T-regulatory cells (Treg) (67,70).

T-helper type 1 (T\(_H\)1) cells, important for immunity to intracellular microorganisms and responsible for the initiation of organ specific autoimmune diseases, produce large amounts of the cytokines interferon-\(\gamma\) (IFN-\(\gamma\)), lymphotoxin-α (LTα), and IL-2 upon their activation (71). IFN-\(\gamma\) is a strong activator of macrophages, inducing their microbicidal activity (72). Upon encounter with antigen and stimulation of TLRs on their surfaces, APCs such as
dendritic cells process and present antigen to naïve T cells while producing abundant amounts of IL-12. This IL-12 directly acts on naïve T cells, inducing Th1 differentiation and the secretion of IFN-γ by NK cells (73).

T-helper type 2 (Th2) cells are responsible for immunity to extracellular parasites such as helminths and nematodes (71). In the last two decades, Th2 responses have also been implicated in the pathogenesis of asthma and allergic disease (74). Th2 cells produce a large variety of cytokines including IL-4, IL-5, IL-9, IL-10, IL-13 and IL-25 (67). IL-4 functions as a positive feedback for the differentiation of Th2 cells (75) and mediates IgE class switching in B cells. IgE then binds FcεRI receptors on basophils and mast cells which induces the release of histamine, IL-4 and IL-13 (76,77). IL-5 has been implicated primarily in eosinophil responses (78) while IL-13 is an effector cytokine for the expulsion of helminths (79). Interestingly, the cytokine IL-10 has suppressive activity towards Th1 cell proliferation and dendritic cell activity (80). The innate factors and APCs that govern Th2 responses are only beginning to emerge, but much debate still remains as to the contribution of each mediator (81) Both basophils and dendritic cells have been shown as the APCs of choice for Th2 responses (82) In 1990, the group of Dr. William E. Paul found that in vitro the differentiation of Th2 cells, which produce large amounts of IL-4, requires an initial source of IL-4 (83) In vivo, this has been proven false, as mice lacking IL-4, IL-4R or STAT6, the major transcription factor in IL-4 signaling, are still able to develop Th2 responses (84). This observation attests to the complex nature of Th2 responses, and suggests that other cell types and cytokines may be involved in initiating and propagating Th2 responses in vivo (85).
Recent evidence has challenged the TH1/TH2 paradigm established by Mossmann and Coffman. TH1 cells were uniquely thought to be responsible for organ specific autoimmunity, yet IFN-γ, IFN-γ-receptor, IL12p35 and IL-12 receptor deficient mice still developed experimental autoimmune encephalomyelitis (EAE) believed previously to be mediated by TH1 polarized T cells (86-88). These observations suggested that TH1 activated T cells are not responsible for the observed autoimmune disease in these mice. Through computational analysis, the novel cytokine IL-23 was discovered which shares its p40 chain with the TH1 cytokine IL-12 (89). IL-23 was subsequently found to activate and expand a unique T cell population that produces large amounts of IL-17 termed TH17 cells (90). Further studies showed that naïve T cells do not bear a receptor for IL-23, suggesting that other cytokines may have a role in the initiation of TH17 responses (91). Transforming growth factor-β (TGF-β) and IL-6, when working in synergy, were found to be important in polarizing naïve CD4+ T cells towards a TH17 phenotype (92-94). Finally, the importance of TH17 cells stems primarily from their production of large amounts of IL-17 and IL-22 following activation. With a very broad tissue distribution of the IL-17 and IL-22 receptors, these cytokines can quickly induce a very powerful tissue inflammatory response (91).

To prevent dissemination to the CNS and death, it is accepted that a protective response in the lungs must occur during primary C. neoformans infection (95). Data from animal models also demonstrates that an intact adaptive T cell immune response is required to survive cryptococcal infection, and is consistent with the inverse correlation between CD4+ T cell numbers in the
HIV/AIDS population and the risk of *C. neoformans* infection (96-98). CD4⁺ T cells have been previously credited with preventing dissemination of *C. neoformans* (96), and were shown to sequester cryptococcal cells in the alveoli within large multinucleated granulomas (99). It was also seen that depletion of CD8⁺ T cells led to the delayed clearance of moderately virulent *C. neoformans* 613D cells from the lungs (100). The outer capsule of Cryptococcus has an important role in both the protection from phagocytosis and protection from B cell mediated antibody responses, although the relative contribution of B cell humoral immunity during *C. neoformans* is still debated (101). Utilizing components of the capsule, mice vaccinated with a glucuronoxylomannan-tetanus toxoid vaccine could clear the organism (102). This suggests a role in immunological memory in fighting *C. neoformans* infection, and CBA mice rechallenged after resolution of a primary *C. neoformans* infection showed hallmarks of immunological memory (103). The elements that contribute to the survival of fungal pathogens including *C. neoformans* in the airways of the host, despite the presence of activated immune cells are not well understood. Numerous investigators now propose that the absence of an adaptive T<sub>H1</sub> response in the airways is what contributes to this observed susceptibility (104).

The shift away from a T<sub>H1</sub> to a T<sub>H2</sub> response can be detrimental to the host as has been seen in *Leishmania major* infection (105) (*Figure 4*). Of the roughly 1 million fungal species worldwide, 80 have been associated with respiratory allergy suggesting that the T<sub>H1</sub>/T<sub>H2</sub>/T<sub>H17</sub> balance may play a role in determining the outcome of a *C. neoformans* infection (106). Mice deficient in the receptor for T<sub>H1</sub> cytokine IFN-γ developed much higher fungal burdens in the brain, lungs and
spleen and were unable to develop a full T\textsubscript{H}1 response required to clear the infection (107). Additionally, a study of human cryptococcal meningitis revealed a correlation in \textit{C. neoformans} clearance and IFN-$\gamma$ levels in cerebral spinal fluid (CSF) (108). In contrast, mice deficient in T\textsubscript{H}2 cytokines IL-4, IL-10 or IL-13 had significantly reduced pulmonary fungal burdens compared to their wild type littermates (109,110). Susceptibility to \textit{C. neoformans} infection has been correlated with IL-5 secretion in the lungs, leading to a predominantly eosinophilic type of inflammation (6). Mice lacking both the hallmark T\textsubscript{H}2 cytokines IL-4 and IL-13 have reduced lung fungal burden, less pulmonary eosinophilia, less serum IgE and a switch from AAM to CAM. Although these mice develop a predominantly T\textsubscript{H}1 response to \textit{C. neoformans} H99 infection, they failed to exhibit reduced fungal dissemination to the brain, and ultimately succumb to death like their wild type counterparts (95).

The role of T\textsubscript{H}17 and Treg cellular responses in the immune response to \textit{C. neoformans} are less well understood (62,110). As was discussed previously, the absence of neutrophilia and ultimately of the T\textsubscript{H}17 response seems to be detrimental to the host. New data however seems to suggest that T\textsubscript{H}17 responses have a limited role during \textit{C. neoformans} infection, as IL-17RA-deficient mice did not have altered survival when infected with \textit{C. neoformans} H99 but did have increased fungal dissemination to the brain (111). These data should nonetheless be interpreted with caution as the receptor subunit IL-17RA is shared by both the receptor for IL-17A and IL-17F (IL-25). Tregs are important for keeping the host immune system in check and maintaining the dynamic balance of T\textsubscript{H}1/T\textsubscript{H}2/T\textsubscript{H}17 (112). Treg cells generated during fungal infection have been shown to play an
important role in limiting inflammation (113). It is believed that Tregs mediate their suppressive effects via the secretion of IL-10 and TGF-β, both of which have been shown to modulate the activation status of CD4⁺ T cells (114). As mentioned previously, mice deficient in IL-10 have better outcomes during *C. neoformans* infection, although this effect is mediated by a reduced T₉₂ response in the lungs without systemic reduction in T₉₂ responses (109). This is contrary to previously demonstrated results, where Tregs seemed to have suppressed systemic allergic responses to inhaled fungal allergens (113). Interestingly, the T₉₁7 and Treg cytokine TGF-β has been found to have a dual role during *C. neoformans* infection in rats. When administered early during the course of infection, overall inflammation and macrophage phagocytosis was reduced leading to increased fungal burdens in these rats. When administered late during infection, TGF-β increased the antifungal capacity of macrophages by upregulating lysozyme production leading to reduced fungal burdens (115). These results suggest a potentially dual role for TGF-β in the capacity of Treg cells to diminish inflammation, and the T₉₁7 cell mediated antifungal response to *C. neoformans*. With seemingly varied contributions from all T helper cell subsets to *C. neoformans* immunity, it is vital that we further explore the delicate balance of these cells during *C. neoformans* infection.

**Epithelial polarizing cytokines and disease**

The airway epithelial lining, once thought as simply a barrier to infection, is now recognized as an essential element of the inducible host defense to infection (116). In healthy individuals, it is accepted that following the inhalation
of fungal cells, growth and dissemination is restricted by the mucous-epithelial barrier and AMs (104). Epithelial cells at mucosal sites are critical in initiating and regulating immunologic homeostasis, and we are only beginning to decipher how this is done (117). Epithelial polarizing cytokines, a novel cytokine population secreted by the mucosal epithelium, including thymic stromal lymphopoietin (TSLP), interleukin-25 (IL-25) and IL-33, have recently been shown to orchestrate T\textsubscript{H}2-associated responses in the gut and lungs (85). Upon secretion, these molecules induce the activation of the mucosal epithelium and associated resident immune cells, such as basophils and dendritic cells (117). Activated dendritic cells then migrate to the draining lymph nodes and present antigen to circulating naïve T cells that differentiate into T\textsubscript{H}2 cells (85). These unique cytokines may therefore have an important role in linking innate and adaptive immunity (118).

These cytokines have been demonstrated to have both protective and pathological roles in disease (85) (Table 1). The T\textsubscript{H}2 cytokine response has been shown to orchestrate protective immunity to helminth infection, all the while being detrimental to the host. To date TSLP has been the best studied of the epithelial polarizing cytokines, following its discovery in the supernatant of cultured thymic stromal cell line (119). The TSLP receptor, consisting of TSLPR and IL-7R\textsubscript{\alpha}, has been shown to be expressed highly on myeloid dendritic cells (120). TSLP-stimulated dendritic cells prime naïve CD4\textsuperscript{+} T cells in an antigen-dependent manner, leading to a T\textsubscript{H}2 activated phenotype (121). Furthermore, TSLP promotes the activation of basophils \textit{in vivo} (82,122). Apart from dendritic cells and basophils, TSLP has also been shown to activate naïve CD4\textsuperscript{+} T cells,
mast cells, NKT cells and eosinophils (123,124). TSLP has been shown in a mouse model of allergic inflammation to be necessary and sufficient for the development of T_{h2} cytokine associated inflammation (125). Additionally, tissue from human asthmatics and patients with atopic dermatitis demonstrate high TSLP expression, further supporting a role for this cytokine in polarizing a T_{h2} response (126,127).

IL-25 is part of the IL-17 family of cytokines and signals via the receptor IL-17RB (128). It primarily induces T_{h2} responses both in vivo and in vitro leading to the production of IL-4, IL-5 and IL-13 and has been shown to be secreted by primed T_{h2} cells (129,130). Furthermore, mice deficient in IL-25 show a poor resistance to the parasites *Trichurus muris* (131) and *Nippostrongylus brasiliensis* (132), further confirming the importance of IL-25 during T_{h2} responses. The study of human tissue has also confirmed the presence of IL-25 in asthmatics (133), patients with rheumatoid arthritis (134), multiple sclerosis (135), or systemic lupus erythematosus (136).

**Interleukin-33**

IL-33, another epithelial polarizing cytokine, was discovered in 2005 as the ligand for the orphan receptor T1/ST2 (IL-1RL1) (137) that is found on T_{h2} cells but not T_{h1} cells (138), dendritic cells (139), mast cells (140), basophils (141), eosinophils (142), neutrophils (143) and as a soluble extracellular form (sST2) (144) (Figure 5). IL-33 is primarily responsible for initiating and amplifying T_{h2} responses via the release of IL-4, IL-5 and IL-13 from these cells (85) and is chemotactic for T_{h2} cells both in vitro and in vivo (145). IL-33 is a
member of the IL-1 family of cytokines that includes IL-1 and IL-18 (146), and was originally found as a nuclear factor associated with human high endothelial venules (HEV) (147). IL-33 is released upon cell necrosis (148) and is believed to be degraded during apoptosis by caspases (149). These lines of evidence and immunostaining of HEV endothelial cell nuclei suggest that IL-33 functions both as an alarmin and transcriptional repressor similar to IL-1α and chromatin associated cytokine HMGB1 (150,151). High IL-33 expression in structural tissue such as endothelial and epithelial cells in atopic dermatitis patients is released into the blood stream following episodes of anaphylactic shock (152,153), fueling the notion that IL-33 acts as an alarmin. IL-33 signals via a dimer of ST2 and the IL-1 receptor accessory protein (IL-1RAP) (154), recruiting the myeloid differentiation primary-response protein 88 (MyD88) complex via the TIR domains of ST2 and IL-1RAP. This in turn triggers the recruitment of IL-1R-associated kinases 1 and 4 (IRAK1 and IRAK4). This culminates in the activation of the transcription factor nuclear factor-κB (NF-κB) via Ca^{2+} mobilization. Alternatively, the MyD88 complex can activate the MAPK kinase pathway leading to the activation of extracellular signal-regulated kinase 1 (ERK1), ERK2, P38 and c-JUN N-terminal kinase-1 (JNK1) (Figure 6). These pathways culminate in Th2 cytokine and chemokine release, mast cell degranulation and production of prostaglandins and leukotrienes (137,152,153).

Interest in IL-33 stems from its wide range of targets in both human and mouse tissues and by the observation that IL-33 plays a dual role in disease by promoting a Th2 host-protective response to helminth infections and atherosclerosis while causing detrimental outcomes in disease models associated
with TH2 immunopathology such as asthma and leishmaniasis (152). Susceptible mice infected with *Trichuris muris*, a nematode parasite, could expel the parasite upon administration of exogenous IL-33 protein leading to a TH2 protective immune response (155). ST2 knockout mice subjected to pressure overload by transverse aortic constriction had reduced survival compared to wild-type controls, suggesting a protective role for IL-33 in the cardiovascular system (156). Blocking ST2 in *Leishmania major* infected Balb/c mice resulted in the development of less severe disease with a TH1 type T cell phenotype (138,152). Research at McGill University has shown that IL-33 is expressed in high levels in the lung tissue of asthmatic patients as compared to healthy individuals (157) and other groups have shown a corresponding elevation in mouse models of asthma (158). Furthermore, IL-33 administered exogenously to mice causes eosinophilia and airway hyper-responsiveness (AHR), classic features of asthma and a TH2 immune response (142).

IL-33 signaling has been implicated in the control of viral infections. ST2 blocking antibody reduced lung inflammation in a mouse model of respiratory syncytial virus infection that is characterized by TH2 immunopathology (159). Ultimately IL-33 can initiate and amplify the adaptive T-helper type 2 (TH2) response (85), but new evidence suggests a role for IL-33 signaling independent of TH2 cells and adaptive immunity. Influenza A infection can worsen asthma symptoms by leading to increased AHR in mice. This effect was recently shown to be orchestrated via a new non-T non-B innate immune cell population coined “natural helper cells” in the lungs (160). IL-33 signaling occurred independently of TH2 cells, and led primarily to IL-13 release by these cells. These findings
further add to the complex pathways of IL-33 signaling \textit{in vivo} and build upon our knowledge of innate lymphoid populations such as nuocytes, IL-25 and IL-33 responsive cells in the gut that are implicated in host protection to helminth infection (161). In response to IL-25 and IL-33 signaling via the IL-17RB and ST2 receptors on the nuocyte surface, these innate lymphoid cells produce large quantities of the cytokines IL-5 and IL-13 (162).

A recently unique role for IL-33 has been uncovered, where IL-33 administration attenuated sepsis in mice (143). Successful clearance of bacterial infection leading to sepsis requires the action of neutrophils (163). Using cecal ligation and puncture as an experimental model of sepsis, exogenous IL-33 administration was able to activate neutrophils while suppressing TLR activation, and was associated with increased survival. Furthermore, patients who had recently died from sepsis were shown to have increased levels of sST2 which leads to a blockade of IL-33 signaling (143). Further study of the highly diverse and very exciting IL-33-ST2 signaling pathway may ultimately provide us with novel therapeutic pathways for a wide range of infectious diseases.

**Interleukin-33 and \textit{C. neoformans} infection in mice**

Fungi produce various enzymes and other mediators that can irritate the respiratory epithelium, leading to the eventual release of a plethora of cytokines and chemokines (104). It is therefore reasonable to postulate that epithelial polarizing cytokines such as IL-33 are released in response to fungal infection. The role of epithelial polarizing cytokines has not been previously investigated in \textit{C. neoformans} infection. Infection of C57BL/6 mice with the moderately virulent
C. neoformans 52D strain or infection of Balb/c mice with the highly virulent C. neoformans H99 strain results in disseminated disease with Th2 immunopathology. Infection of the resistant mouse strains CBA and SJL with C. neoformans 52D results in less severe disease with a Th1 immunophenotype (164). Mice deficient in both IL-4 and IL-13 have been shown to have less severe lung pathology, yet this does not provide them with a significant survival advantage as they succumb to infection following C. neoformans dissemination to the brain (95). This suggests that other factors may be associated with susceptibility to lethal cryptococcal infection. Huffnagle and McNeil state that the mechanisms associated with C. neoformans clearance are similar in both the lungs and brains of mice (43). As Il33 mRNA is highly expressed in the lungs, spleen, and brains of mice (137), it may contribute to the development of a Th2 phenotype that is associated with susceptibility to C. neoformans infection. Furthermore, alveolar macrophages important in the response to C. neoformans, have recently been shown to release IL-33 upon influenza infection (160). One group also recently demonstrated that the fungal aeroallergen Alternaria alternata, triggers the release of IL-33 in the airways of mice (165).

We chose to investigate the contribution of IL-33 to immunity in C. neoformans infection to better determine what is driving the detrimental Th2 allergic response observed in mice, and if indeed allergic Th2 responses are pathological or if it is simply a lack of Th1 sterilizing immunity that predisposes to disease. We hypothesize that, in addition to IL-4 and IL-13, the release of the epithelial polarizing cytokine IL-33 during C. neoformans infection triggers
allergic lung inflammation that confers susceptibility to progressive pneumonia, meningitis, and disseminated fungal disease.
Figure 1: The Cryptococcus species complex. The Cryptococcus species complex can be divided into two distinct subtypes, now recognized as unique species. *Cryptococcus neoformans* is ubiquitous in the environment and infects immunocompromised individuals. *Cryptococcus gattii* is found in tropical and subtropical regions, however recently it has been found as far north as Vancouver Island, British Columbia, Canada. The two subtypes can be further divided into 4 serotypes and 9 distinct molecular types (*not shown*). Figure was adapted from Lin et al., 2006 (23).
Figure 2: Cryptococcal spore acquisition from the environment. Pigeons and birds are large contributors to cryptococcal biomass in the environment as *Cryptococcus* readily multiplies in their guts and is released via their excreta. Other animal hosts, including cows, cats, guinea pigs, insects and even unicellular amoebas can be infected by *Cryptococcus* and contribute to cryptococcal cell shedding into the environment. *Cryptococcus* can also be readily found on rotting vegetables and fruits, rotting wood, eucalyptus trees and in the soil. Airborne spores or yeasts are inhaled by humans, resulting in cryptococcal pneumonia. This can lead to fungal dissemination to the brain, cryptococcal meningitis and death if left untreated. Figure was adapted from *Lin et al.*, 2006 (23).
Figure 3: Mouse strain survey for *C. neoformans* 52D susceptibility. Mice were infected intratracheally with $1 \times 10^4$ CFU of *C. neoformans* 52D and lungs were harvested at 5 weeks post infection. Each point represents the total lung CFU for an individual mouse. Results and figure kindly provided by Scott Carroll.
**Figure 4:** T-helper cell response to *C. neoformans* determines host susceptibility. The T-helper cell response mounted by the host determines the susceptibility to *C. neoformans* infection. A TH1 and TH17 type of response is generally protective whereas a TH2 response is detrimental. Not enough data is currently available on the T regulatory response to *C. neoformans*.
**Figure 5: IL-33 acts upon a broad spectrum of cells.** The epithelial lining, and alveolar and peritoneal macrophages have been previously demonstrated to secrete IL-33 in response to allergens, helminths and influenza virus. IL-33 acts on Th2 cells, basophils (BP), eosinophils (EO), mast cells (MC), NK and NKT cells (NK/NKT), neutrophils (NT), dendritic cells (DC) and a broad category of cells termed natural helper cells (NHC) that include but are not limited to nuocytes.
Figure 6: The proposed IL-33 signaling pathway. Following necrosis, IL-33 is released from cells and can be neutralized by the soluble form of the ST2 receptor (sST2) or it can signal via a dimer of ST2 and IL-1 receptor accessory protein (IL-1RAP). Signal transduction proceeds via TIR domain recruitment of MyD88 leading to Ca^{2+} mobilization and subsequent activation and nuclear translocation of NFκB or the activation of MAPK kinases (MAPKKs), ERK, p38 and JNK leading to eventual gene transcription. These pathways culminate in mast cell degranulation, cytokine and chemokine release, and the production of prostaglandins and leukotrienes. Figure was adapted from Liew et al., 2010 (152).
Table 1: The protective and pathological roles of the epithelial polarizing cytokines TSLP, IL-25 and IL-33 in disease.

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<th>TSLP</th>
<th>IL-25</th>
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<td><strong>Protective</strong></td>
<td>- Mice deficient in TSLPR were unable to control <em>Trichuris muris</em> infection</td>
<td>- IL-25 administration to <em>Trichuris muris</em> infected mice helped expel the parasite (131)</td>
<td>- IL-33 administration to <em>Trichuris muris</em> infected mice helped expel the parasite (155)</td>
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<td>- Mice deficient in IL-25 display inefficient <em>Nippostrongylus brasiliensis</em> expulsion (130)</td>
<td>- IL-33 administration attenuated sepsis in mice (143)</td>
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<td>- IL-33 is protective in the cardiovascular system (156)</td>
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<td><strong>Pathological</strong></td>
<td>- Human asthmatics have high TSLP expression in the lungs (127)</td>
<td>- Overexpression and exogenous administration of IL-25 in mice led to allergic pathologies (129,130)</td>
<td>- High IL-33 expression in human asthmatic airways (157)</td>
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<td>- Atopic dermatitis development in mice with a TSLP transgene (167)</td>
<td>- High IL-25 expression in human asthmatic airways (133)</td>
<td>- IL-33 administration to mice leads to an asthmatic phenotype (142)</td>
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<td>- TSLPR deficient mice are resistant to OVA induced asthma (125,168)</td>
<td>- Elevated levels of IL-25 in rheumatoid arthritis (134), multiple sclerosis (135) and systemic lupus erythematosus (136)</td>
<td>- Blocking ST2 helped control respiratory syncytial virus in mice (159)</td>
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<td>- Blocking ST2 led to less severe Leishmaniasis in mice (138)</td>
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MATERIALS AND METHODS

**Mice**

All experimental protocols were reviewed and approved by the McGill University Animal Care Committee. Wild type six-week-old female Balb/c, C57BL/6 (Charles River, Saint-Constant, QC) and CBA (Harlan Laboratories, Indianapolis, IN) mice were purchased and maintained in our facility, provided with sterile food and water *ad libitum*, and cared for according to the Canadian Council on Animal Care guidelines. T1/ST2−/− mice were bred on site, kindly provided by Dr. Jorg Fritz, and originally generated in the lab of Dr. Andrew McKenzie (169). Mouse genotypes were verified using ear tissue and a PCR reaction using the T1/ST2 primers (5’ – TTG GCT TCT TTT AAT AGG CCC – 3’) and (5’ – TGT TGA AGC CAA GAG CTT ACC – 3’) and the following cycling conditions: denaturation at 94°C for 5 mins, 35 cycles at 94°C for 1 min, 61°C for 1 min, and 72°C for 1 min. Infections were performed with six-to-seven-week-old T1/ST2−/− mice using age and sex matched Balb/c mice as controls, and mice were humanely euthanized with CO2 upon completion of experiments.

** Cultures of *C. neoformans***

*C. neoformans* H99 (ATCC no. 208821), 52D (ATCC no. 24067) and CAP64 (ATCC no. 52816) were thawed from glycerol stocks and maintained on Sabouraud dextrose agar (SDA) (BD, Sparks, MD). A single colony of *C. neoformans* was resuspended in Sabouraud dextrose broth at a starting concentration of 5 x 10^5 yeasts/mL, and grown in a rotating culture until
stationary phase (48 hours) either at room temperature for *in vivo* infections or at 37°C for *in vitro* stimulations. Subsequently the culture was centrifuged, washed twice with PBS, and resuspended to a desired concentration in PBS or DMEM (Gibco, Dorval, QC) supplemented with 2% Fetal Bovine Serum (FBS) (Wisent, Québec, QC). Concentrations of *C. neoformans* were verified by plating on SDA at 37°C for 72 hours and subsequently counting the colony-forming units (CFU).

**Intratracheal and intranasal infection with *C. neoformans***

For intranasal (IN) instillation of *C. neoformans, C. neoformans* H99 and 52D were diluted to $2 \times 10^5$ CFU/mL and $2 \times 10^7$ CFU/mL in PBS, respectively. Mice were lightly anesthetized with 5% isofluorane, hung vertically from a surgical board, and 50μL of the *C. neoformans* mixture was pipetted into their nares. For intratracheal (IT) instillation of *C. neoformans*, mice were anesthetized with 10mg/kg of ketamine (Ayerst Veterinary Laboratories, Guelf, ON) and 125mg/kg of xylazine (Bayer Inc, Pittsburgh, PA) intraperitoneally (IP). A vertical 2cm incision was made below the jaw along the trachea, and the underlying glands and smooth muscle were pushed aside. A 22-gauge catheter (BD, Sparks, MD) was inserted into the trachea, and in a volume of 50μL of PBS, $2 \times 10^5$ CFU/mL of *C. neoformans* H99 or 52D was instilled followed quickly by a 50μL volume of air. The incision was closed using a 9mm EZ Clip wound closing kit (Stoelting, Wood Dale, IL). Mice were monitored daily following surgery.
Organ isolation and CFU assay

Following euthanasia by CO₂, mice were immersed in 70% ethanol, subjected to terminal cardiac puncture, and dissected along the midline using sterile technique. Lungs, spleen and brain were excised and placed into separate tubes containing sterile PBS on ice. Subsequently, the organs were homogenized using a sterilized glass tube and pestle attached to a mechanical tissue homogenizer (Glas-Col, Terre Haute, IN) and plated at various dilutions on SDA. Plates were incubated at 37°C for 72 hours and CFU were counted.

Bronchoalveolar lavage

Mice were euthanized using CO₂, immersed in 70% ethanol, and an incision was made below the jaw to expose the trachea. A 22-gauge catheter was inserted into the airway and secured in place by a string. A total of 4 volumes of 500μL of ice-cold PBS were instilled via the catheter and subsequently extracted for a total of 2mL. The bronchoalveolar lavage fluid was then centrifuged at 1200rpm for 10 minutes and the supernatants were stored for subsequent analysis.

MLE-12 mouse lung epithelial cell line studies

MLE-12 cells (ATCC no. CRL-2210) were grown to 80% confluence in DMEM (Wisent, Québec, QC) supplemented with 2% FBS, 1% L-glutamine (Gibco, Dorval, QC) and 1% penicillin and streptomycin (Gibco, Dorval, QC) at 37°C in a 5% CO₂ atmosphere as per ATCC recommendations. For passaging, cells were washed with sterile PBS, subjected to incubation with 0.25% trypsin (Gibco, Dorval, QC) and scraped from the bottom of the flask using a cell scraper (BD, Sparks, MD). Cells were counted using a Beckman Coulter cell counter.
(Beckman Coulter, Mississauga, ON), plated at 170 000 cells per well in a 6-well plate, and grown for 48 hours to 100% confluence. Subsequently, cells were stimulated with increasing multiplicities of infection (MOI) of \textit{C. neoformans} H99, 52D or CAP64 and incubated for 3, 6 or 24 hours at 37°C in a 5% CO\textsubscript{2} atmosphere. Supernatant was collected and stored at -80°C for future analysis.

\textit{In vitro and lung mRNA expression}

For \textit{in vitro} studies, cells were scraped from the plate, disrupted using lysis buffer, and homogenized using a QIAShredder column. Total RNA was extracted using the RNeasy Plus kit (Qiagen, Mississauga, ON). For lung RNA extraction, 4mm of lung was collected in RNAlater solution (Invitrogen, Grand Island, NY), homogenized in lysis buffer using a tissue homogenizer (Fisher Scientific, Sainte-Foy, QC) and RNA was extracted using an RNeasy kit. Using 0.4µg of RNA, a reverse transcription reaction was performed using an ABI high capacity cDNA reverse transcription kit (ABI, Foster City, CA). Real-time quantitative PCR (RT-qPCR) was performed with an ABI Prism 7500 Real time PCR system (ABI, Foster City, CA). Each reaction well contained 10µL TaqMan Genotyping Master Mix (ABI, Foster City, CA), 5µL RNase-free water (Wisent, Québec, QC), 1µL of Taqman probes (ABI, Foster City, CA) and 40ng of cDNA template in 4µL of water. The probes used were \textit{Il25} (Mm00499822_m1), \textit{Il33} (Mm00505403_m1), \textit{Tslp} (Mm00498739_m1), \textit{Il4} (Mm00445258_g1), \textit{Il5} (Mm00439646_m1), \textit{Il13} (Mm00434204_m1), \textit{Ifnγ} (Mm99999071_m1), and \textit{β-actin} (Part no. 401846) as a housekeeping gene. Results were calculated using the change-in-cycling-threshold
method ($2^{\Delta\Delta C_t}$) relative to the expression of $\beta$-actin and presented as a fold induction relative to unstimulated samples.

**Total lung protein isolation**

Mice were euthanised and lungs flushed with 10mL of ice-cold PBS. Whole lungs were homogenized in 2mL PBS with Halt Protease and Phosphatase Inhibitor Cocktail (Fisher Scientific, Sainte-Foy, QC) using a sterilized glass tube and pestle attached to a mechanical tissue homogenizer and centrifuged at 12,000rpm for 20 minutes. Supernatants were collected and stored at -80°C until analysis.

**Cytokine production**

Cell culture supernatants, bronchoalveolar lavage fluid or whole lung protein samples were assayed using DuoSet ELISA kits (R & D Systems, Minneapolis, MN) for IL-33 (Catalog no. DY3626) and IL-25 (Catalog no. DY1399) as per the manufacturer’s protocol.

**Calculations and statistical analysis**

For all experiments the mean and standard error of means (SEM) are shown. All figures show the results from a single experiment, and explicitly state how many times these results were replicated. To test the significance of single comparisons, an unpaired Student’s t-test was applied with a threshold P value of < 0.05. Multiple comparisons were tested using a one-way ANOVA with either a Dunnett post-test for comparison of individual means to a control or a Bonferroni
post-test for comparison of all individual means within a figure. For all tests, * denotes p<0.05, ** denotes p<0.01 and *** denotes p<0.001.
RESULTS

Acapsular *C. neoformans* induces *Il33* mRNA expression *in vitro* in a mouse lung epithelial cell line

Our primary goal was to show that epithelial polarizing cytokines are induced in response to *C. neoformans* infection. Initially, we tested the immortalized mouse lung epithelial cell line MLE-12 cultured *in vitro* at 37°C and 5% CO₂. It was previously established in our laboratory that the human bronchial epithelial cell line (BEAS-2B) was most strongly activated by the capsule-deficient mutant strain of *C. neoformans* (CAP64) grown in a rotating culture at 37°C (55). For this reason, MLE-12 cells were plated at 170 000 cells per well, grown to confluence for 48 hours, and infected with increasing multiplicities of infection (MOI) of CAP64 for either 3 or 24 hours. Positive controls included papain (170) and poly I:C (*data not shown*); both compounds were previously shown to strongly induce *Tslp* in human airway epithelial cells and papain was also shown to strongly induce *Il25* in MLE-12 cells (172). At each experimental endpoint, the cell supernatants were collected and frozen for later use and total cellular RNA was extracted using a commercial kit. Real-time PCR analysis was subsequently performed to determine the fold-induction of the epithelial polarizing cytokines *Tslp, Il25,* and *Il33* relative to an unstimulated control after normalization to β-actin (*Figure 7*). Upon analysis of the results, no *Tslp* induction was detectable in any of the samples (*Figure 7A and 7B*). Further analysis of the results demonstrated an early, though not significant, induction of *Il25* mRNA (2.08 ± 1.14 fold) (*Figure 7C and 7D*) and a later significant
induction of \( I\!l_{33} \) mRNA (8.58 ± 1.90 fold) at an MOI of 50 (Figure 7E and 7F). Cell culture supernatants were positive for the secretion of IL-8 detected by an enzyme-linked immunosorbent assay (ELISA) which confirmed that \( C.\ neoformans \) was indeed activating MLE-12 cells (data not shown). Consistent with previous findings, IL-33 protein in cell culture supernatants could not be detected by ELISA (158) (data not shown). These findings in MLE-12 cells give us the first evidence that epithelial polarizing cytokines can be expressed in response to \( C.\ neoformans \) infection.

**Highly virulent \( C.\ neoformans \) induces the expression of \( I\!l_{25} \) and \( I\!l_{33} \) in a mouse lung epithelial cell line**

To extend these in vitro findings, we infected MLE-12 cells with the serotype A \( C.\ neoformans \) H99 strain. This encapsulated human isolate is highly virulent in mouse models of infection (173,174) and promotes a shift to non-protective T\(_h\)2 immunity in Balb/c inbred mice. Infection of MLE-12 cells with \( C.\ neoformans \) H99 triggered significant time- and dose-dependent increases in \( I\!l_{25} \) and \( I\!l_{33} \) mRNA expression (Figure 8). After cryptococcal stimulation, a 2.93 ± 0.31 (Figure 8A) and 7.25 ± 0.76 (Figure 8B) fold induction of \( I\!l_{25} \) was observed at 3 and 6 hours, respectively, while a 4.93 ± 0.33 (Figure 8E) and 3.13 ± 0.16 (Figure 8F) fold induction of \( I\!l_{33} \) was observed at 6 and 24 hours post-infection, respectively. Again no IL-33 protein was detected in the cell culture supernatants (data not shown). These results further provide evidence that a virulent clinical isolate of \( C.\ neoformans \) can induce epithelial polarizing cytokine expression.
IL-33 protein and mRNA expression in wild type Balb/c mice following *C. neoformans* H99 infection

Following these initial positive *in vitro* results using *C. neoformans* H99, we decided to test for epithelial polarizing cytokine expression in a clinically relevant mouse model of *C. neoformans* pulmonary infection. In our initial experiment, 7 week-old Balb/c mice were intranasally infected with $1 \times 10^4$ CFU of *C. neoformans* H99 for 3, 7 or 14 days as previously described (175). Lungs were harvested, homogenized and mRNA was extracted and quantified (*Figure 9A-C*). Real-time PCR analysis did not show *Tslp* or *Il25* expression (*Figure 9A and 9C*). Significant *Il33* expression was evident in a time-dependent manner with $1.28 \pm 0.13$, $2.54 \pm 0.99$ and $4.31 \pm 0.43$ fold at 3, 7 and 14 days, respectively (*Figure 9B*). Furthermore, whole lung protein analysis of IL-33 showed a trend towards higher IL-33 protein at later time points, although the differences were not significant (*Figure 9E*). IL-25 protein in whole lung homogenates was not clearly visible, corroborating the mRNA results (*Figure 9D*). Taken together, these results provide the first evidence that IL-33 can be expressed *in vivo* in response to pulmonary *C. neoformans* infection.

**Differential *Il33* expression in susceptible vs. resistant stains of wild type mice in response to intranasal *C. neoformans* infection**

IL-33 has been mainly associated with $T_\text{H}2$ responses both *in vitro* and *in vivo* (152). Interestingly, in response to *C. neoformans* 52D infection, susceptible C57BL/6 mice develop a $T_\text{H}2$ type of response upon while resistant CBA mice develop a strong $T_\text{H}1$ response. We therefore postulated that differential *Il33*
mRNA expression would be demonstrable between these two strains of mice. To test this possibility, 7 week-old C57BL/6 and CBA mice were intranasally infected with $1 \times 10^6$ CFU of *C. neoformans* 52D as previously described (49), and seven or fourteen days later these mice were sacrificed and their lungs harvested (*Figure 10*). As expected, CBA mice had a significantly lower lung fungal burden at 7 and 14 days post infection as compared to C57BL/6 mice (*Figure 10A*). Subsequent lung mRNA analysis showed no induction of *Tslp* (*data not shown*) and no significant *Il25* induction in either strain (*Figure 10B and 10C*). Interestingly, CBA mice showed a trend towards increased *Il25* mRNA expression; however, these results were not significant (*Figure 10C*). Additionally, RT-qPCR analysis did not show any significant increase in *Il33* mRNA expression in the resistant CBA strain (*Figure 10E*). RT-qPCR analysis demonstrated a $3.19 \pm 0.28$ and $1.73 \pm 0.32$ fold induction of *Il33* mRNA at 7 and 14 days, respectively in susceptible C57BL/6 mice (*Figure 10D*). The results demonstrate differential *Il33* expression among resistant and susceptible strains of mice following *C. neoformans* infection that is consistent with its role in T<sub>H2</sub> immune responses.

**Differential *Il33* expression among wild type mice infected with moderate or highly virulent strains of *C. neoformans***

It was previously shown that Balb/c mice infected with *C. neoformans* H99 have a T<sub>H2</sub> pattern of cytokine expression and generally succumb to infection by 4 weeks (174). Interestingly, when infected with the moderately virulent *C. neoformans* 52D, these mice show an intermediate resistance to lung fungal burden, between that of C57BL/6 and CBA mice (*Figure 3*). Based on these
observations, we investigated $Il33$ mRNA and protein expression in Balb/c mice following $C. neoformans$ H99 or 52D infection. As expected, Balb/c mice infected with 52D have a decreasing fungal burden following 7 days of infection, whereas the lung fungal burden continued to rise significantly following infection with H99 (Figure 11A). Analysis of lung $Il33$ mRNA expression also yielded significantly different results following infection with these two $C. neoformans$ strains (Figure 11B). At 7 days post infection, Balb/c mice infected with 52D had $0.74 \pm 0.11$ fold induction of $Il33$ mRNA, whereas mice infected with H99 had $2.543 \pm 0.99$ fold induction of $Il33$. At 14 days the difference in $Il33$ mRNA induction was greater with $0.43 \pm 0.09$ and $4.31 \pm 0.43$ fold induction for 52D and H99 infected mice, respectively.

Interestingly, this effect is not limited to Balb/c mice. CBA mice show a significantly higher fungal burden at 14 days post-infection with $C. neoformans$ H99 compared to $C. neoformans$ 52D (Figure 12A) that correlates with $Il33$ mRNA expression. $C. neoformans$ H99 infection induced $1.23 \pm 0.14$ and $1.82 \pm 0.30$ fold $Il33$ mRNA expression at 7 and 14 days respectively, compared to $0.72 \pm 0.09$ and $0.79 \pm 0.29$ fold induction among 52D infected mice on the same days (Figure 12B). To further confirm a Th2 pattern of response in the CBA mice, mRNA expression of $Ifn\gamma$, $Il4$, $Il5$ and $Il13$ were measured in lung tissue (Figure 12C-F). Mice infected with $C. neoformans$ 52D had significantly higher mRNA expression of the Th1 mediator $Ifn\gamma$ at 14 days post infection (Figure 12C), whereas mice infected with $C. neoformans$ H99 had significant higher mRNA expression of the Th2 mediators $Il4$, $Il5$ and $Il13$ (Figure 12D-F). Taken together, these results show that $Il33$ mRNA induction depends both on the strain of $C.$
*C. neoformans* used for infection and the mouse genetic background, and that the mRNA expression level correlates with a predominantly T$_{h2}$ cytokine milieu.

**Decreased fungal burden in mice devoid of IL-33 signaling**

With prior results demonstrating an increased abundance of IL-33 protein and mRNA in Balb/c mice infected with *C. neoformans* H99, we wanted to determine the effect of abolished IL-33 signaling in this mouse model of infection. Accordingly, we obtained mice that are deficient for T1/ST2, the receptor for IL-33, on the Balb/c background. Mice were infected with $1 \times 10^4$ CFU of *C. neoformans* H99 intratracheally as previously described (22) and were monitored for 3 weeks at which point they were sacrificed and their lung, spleen and brain fungal burdens were assessed (*Figure 13*). Consistent with previously published results the wild type mice had $4.62 \times 10^8 \pm 1.19 \times 10^8$ CFU per lung, while mice lacking T1/ST2 dependent signaling had a significantly lower fungal burden of $1.35 \times 10^8 \pm 3.46 \times 10^7$ CFU per lung (*Figure 13A*). Furthermore, the spleens of mice lacking T1/ST2 showed a significantly reduced CFU in the knockout mouse, with wild type mice having $6.12 \times 10^5 \pm 2.28 \times 10^5$ CFU versus knockouts having $1.56 \times 10^4 \pm 8.60 \times 10^3$ CFU per spleen (*Figure 13B*). Finally, a significant difference in brain CFU was observed between both mouse groups 3 weeks post-infection with wild type mice having $1.07 \times 10^7 \pm 6.67 \times 10^5$ CFU per brain and knockout mice having $1.13 \times 10^6 \pm 6.92 \times 10^5$ CFU per brain (*Figure 13C*). Overall these data support a novel and detrimental role for IL-33 signaling following pulmonary *C. neoformans* infection.
Figure 7: Capsule independent induction of epithelial polarizing cytokine mRNA in cultured mouse lung epithelial cells stimulated with *C. neoformans*. MLE-12 cells were grown to confluence and were either stimulated with media (NS), papain (0.25mg/mL), or *C. neoformans* CAP64 at a multiplicity of infection (MOI) of 100, 50, 25 and 10 for 3 hours or 24 hours. Total mRNA was extracted, reverse transcribed and gene expression normalized to β-actin using 2^{-ΔΔCt} method. *Tslp* (A-B), *Il25* (C-D) and *Il33* (E-F) mRNA expression is expressed as n-fold induction over media stimulated cells. Data from one experiment are expressed as means ± SEM for each time point with 2 technical and 3 biological replicates per group. *P<0.05, **P<0.01 and *** P<0.001 using a one-way ANOVA analysis with Dunnett post-test comparing all sample means to the media stimulated cell median.
**Figure 8:** IL25 and IL33 mRNA induction following the stimulation of cultured mouse lung epithelial cells with highly virulent *C. neoformans*. MLE-12 cells were grown to confluence and were either stimulated with media (NS), papain (0.25mg/mL), or *C. neoformans* H99 at a multiplicity of infection (MOI) of 100, 50, or 10 for 3 hours, 6 hours or 24 hours. Total mRNA was extracted, reverse transcribed and expression normalized to β-actin using 2^{-ΔΔCt} method. IL25 (A-C) and IL33 (D-F) mRNA expression is expressed as n-fold induction over media stimulated cells. Data from one experiment are expressed as means ± SEM for each time point with 2 technical and 3 biological replicates per group. *P<0.05, **P<0.01 and ***P<0.001 using a one-way ANOVA analysis with Dunnett post-test comparing all sample means to the media stimulated cell median.
Figure 9: Lung Tslp, Il25 and Il33 mRNA expression in wild type mice following C. neoformans H99 infection. Inbred Balb/c mice were infected intranasally with $10^4$ CFU of C. neoformans H99. After 3, 7 or 14 days lung mRNA was extracted, reverse transcribed and target gene expression was normalized to β-actin using $2^{-\Delta\Delta C_{t}}$ method (A-C). RNA expression is shown as n-fold induction over PBS infected animals. ELISAs for IL-25 and IL-33 were performed on lung protein with results expressed as total protein per lung (D-E). Data from one experiment are expressed as means ± SEM for each time point with ≥3 animals per group. ***P<0.001 using a one-way ANOVA with Dunnett post-test comparing infected to PBS treated animals.
Figure 10: Comparison of lung Il25 and Il33 mRNA expression in resistant and susceptible wild type mice following C. neoformans 52D infection. Wild type C57BL/6 and CBA mice were infected intranasally with $10^6$ CFU of C. neoformans 52D for 7 or 14 days. Total CFU per lung was enumerated by plating on Sabouraud dextrose agar at weekly intervals (A), with *P<0.05 and ***P<0.001 using an unpaired Student’s t-test. Lung mRNA was extracted from C57BL/6 and CBA mice, reverse transcribed and gene expression normalized to β-actin using $2^{-ΔΔCt}$ method. Total RNA expression for Il25 (B-C) and Il33 (D-E) is expressed as n-fold induction over PBS infected animals. Data from one experiment are expressed as means ± SEM for each time point with 4 animals per group. ***P<0.001 and no significance (NS) using a one-way ANOVA analysis with Dunnett post-test comparing infected to PBS treated animals.
Figure 11: Comparison of lung *Il33* mRNA expression in Balb/c mice infected with a moderate or highly virulent strain of *C. neoformans*. Wild type Balb/c mice were infected intranasally with $10^6$ CFU of moderately virulent *C. neoformans* 52D or $10^4$ CFU of highly virulent *C. neoformans* H99 for 7 or 14 days. Total CFU per lung were enumerated by plating on Sabouraud dextrose agar at weekly intervals (A). Animals were pooled from separate matched experiments with **P<0.01 and ***P<0.001 using an unpaired Student’s t-test. Lung mRNA was extracted, reverse transcribed and gene expression normalized to β-actin using $2^{\Delta\Delta Ct}$ method (B). Total RNA expression is expressed as n-fold induction over PBS infected animals. Data from one experiment replicated twice are expressed as means ± SEM for each time point with ≥3 animals per group. *P<0.05 and ***P<0.001 using a one-way ANOVA analysis with a Bonferroni post-test correction.
Figure 12: Comparison of lung Il33 mRNA expression in CBA mice infected with a moderate or highly virulent strain of C. neoformans. Wild type CBA mice were infected intranasally with $10^6$ CFU of moderately virulent C. neoformans 52D or $10^4$ CFU of highly virulent C. neoformans H99 for 7 or 14 days. Total CFU per lung were enumerated by plating on Sabouraud dextrose agar at weekly intervals (A). Animals were pooled from separate matched experiments with **P<0.01 and ***P<0.001 using an unpaired Student’s t-test. Lung mRNA was extracted, reverse transcribed and gene expression normalized to $\beta$-actin using $2^{-\Delta\Delta C_T}$ method. Total RNA expression is expressed as n-fold induction over PBS infected animals. Il33 expression (B) after 7 and 14 days infection and Ifn$\gamma$, Il4, Il5 and Il13 expression (C-F) after 14 days infection. Data from one experiment replicated twice (A-B) or once (C-F) are expressed as means ± SEM for each time point with $\geq$3 animals per group. *P<0.05, **P<0.01 and ***P<0.001 using a one-way ANOVA with a Bonferroni post-test correction.
Figure 13: Mice lacking T1/ST2-dependent signaling have reduced lung fungal burden. Balb/c wild type mice and mice lacking the IL-33 receptor T1/ST2 were infected with $10^4$ CFU of *C. neoformans* H99. Following 21 days of infection, lungs (A), spleens (B), and brains (C) were harvested. Total CFU were enumerated by plating on SDA. Data from one experiment replicated twice are expressed as means ± SEM for each time point with wild type mice, n=3; and T1/ST2$^{-/-}$ mice, n=7. **P<0.01 and ***P<0.001 using an unpaired Student’s t-test analysis.
DISCUSSION

**Initial evidence for the role of IL-33 during *C. neoformans* infection**

From our research, we have identified a novel role for IL-33 during *C. neoformans* infection by: 1) demonstrating its upregulation at the mRNA level in a mouse lung epithelial cell line in response to *C. neoformans* infection; 2) showing its differential mRNA expression in susceptible and resistant strains of mice during *C. neoformans* infection; and 3) demonstrating that mice lacking ST2-dependent signaling have a reduced lung, spleen and brain fungal burdens.

Our current knowledge of infectious diseases suggests that not only is the microbial agent an important determinant of the outcome, but that the host immune response to that microbe also plays a crucial role. Numerous observations suggest that an allergic type of response is detrimental to the host during *Cryptococcus neoformans* infection. Nevertheless, it is not completely clear whether a T\(_{H2}\) response to *C. neoformans* is the primary factor that leads to poor outcomes, or if the lack of T\(_{H1}\) sterilizing immunity forces the host to mount an ineffective T\(_{H2}\) attack that is too weak to clear the infection. Thus, many investigators would like to know what provokes the allergic response to *C. neoformans* and whether its elimination would be beneficial to the host.

The role of IL-33 in allergic inflammation is only beginning to be deciphered. Many cellular sources and targets have been found to both produce and respond to IL-33, making the prospect of elucidating a new specific pathway quite exciting (*Figure 5*). The role of the respiratory epithelium during infection is not only that of a physical barrier to infection, but has been demonstrated to
secrete a large array of inflammatory mediators and antimicrobial factors important to host innate immunity. Using human bronchial epithelial cell lines and alveolar macrophages, our group and others have demonstrated that these cell types respond to *C. neoformans* infection *in vitro* by phagocytosing *C. neoformans* or by producing large amounts of the inflammatory mediator IL-8 (55,176,177). We therefore wanted to investigate if other airway epithelial factors play a role in susceptibility and resistance to *C. neoformans* infection. Epithelial polarizing cytokines are a recently identified class of mediators released by the airway epithelium that have been demonstrated to stimulate TH2 responses both *in vivo* and *in vitro* in helminth and asthma models (117). Using a capsule deficient mutant of *C. neoformans* that was previously found to be a strong inducer of airway epithelial cell activation, we demonstrate an induction of *Il33* mRNA following 24 hours of stimulation that was further confirmed using the encapsulated highly virulent human isolate *C. neoformans* H99 (*Figure 7 and 8*). Although the direct release of IL-33 protein *in vitro* from the cells could not be demonstrated, these results suggest that upregulation of *Il33* mRNA can occur following damage to the epithelial lining and is consistent with the previous observation that IL-33 may be released following necrosis of these cells (151). Using the release of lactate dehydrogenase (LDH) as a marker of cellular damage, we previously demonstrated that BEAS-2B epithelial cells are damaged following *C. neoformans* infection, providing indirect evidence that the release of IL-33 from these cells might also be possible (55). In response to *C. neoformans* we show early *Il25* expression, but unexpectedly we could not demonstrate *Tslp* expression in these cells (*Figure 7 and 8*). The use of papain and poly I:C as
positive controls also stimulated *Il25* and *Il33* expression but failed to trigger *Tslp* expression in MLE-12 cells. Previously, *Tslp* expression was demonstrated in a human bronchial epithelial cell line (BEAS-2B) following administration of papain, but this phenomenon may not be generalizable to MLE-12 cells (170). Future studies could attempt to use other positive controls in order to elicit *Tslp* expression in these cells.

Previous studies using an intratracheal mouse infection model with the highly virulent *C. neoformans* H99 strain have clearly demonstrated that susceptible Balb/c mice mount a highly polarized T\(_{H2}\) response in the lungs (174). These mice develop severe pulmonary eosinophilia, high serum IgE, high T\(_{H2}\) cytokines such as IL-4 and IL-13 relative to the T\(_{H1}\) cytokine IFN-\(\gamma\) and had detrimental AAM. Upon intranasal infection of Balb/c mice with *C. neoformans* H99, we show both IL-33 mRNA and protein expression in whole lung tissue (*Figure 9*). It is currently understood that full length IL-33 is released following necrosis, whereas a cleaved form is released following apoptosis (151). IL-33 protein was not detectable in the BAL, suggesting that it may not be released into the airways in this experimental model. In contrast, a recent study using an extract from the fungal allergen *Alternaria alternata* was able to demonstrate IL-33 protein in the BAL (165) and this extract could serve as a positive control in future studies. A drawback of our analysis is that we could not distinguish intracellular and extracellular IL-33, as the homogenization process lysed lung cells and exposes intracellular as well as that secreted proteins. Future studies using immunohistochemistry or laser capture microdissection could provide more conclusive evidence as to the cellular sources of IL-33 protein during
cryptococcal infection. Furthermore, current evidence suggests that IL-33 is differentially processed depending on if release occurred following apoptosis or necrosis (148,149), adding a further level of complexity to our future studies.

One of our most interesting findings is the differential mRNA expression of \textit{Il33} in various strains of inbred mice. Based on analysis of lung fungal burden it has been established that certain mouse strains are more susceptible to the moderately virulent \textit{C. neoformans} 52D than others (Figure 3). We utilized this information to infect highly susceptible C57BL/6 mice and relatively resistant CBA mice with \textit{C. neoformans} 52D. Our results revealed that the C57BL/6, which mounts a \textit{T} \text{H}2 skewed response, had significantly increased \textit{Il33} expression in the lungs compared to CBA, which mounts a \textit{T} \text{H}1 skewed response (Figure 10). Future experiments are currently underway to confirm observed \textit{Il33} mRNA expression by analyzing whole lung protein. These current results demonstrate and provide the first indication that \textit{Il33} expression following \textit{C. neoformans} infection occurs primarily in mice that mount a \textit{T} \text{H}2 pattern of adaptive immune response.

An article by Jain et al. showed that Balb/c mice infected with \textit{C. neoformans} 52D or H99 developed differential \textit{T} \text{H}1 and \textit{T} \text{H}2 responses, respectively (164) This observation that an inbred strain can mount either a \textit{T} \text{H}1 or a \textit{T} \text{H}2 response, depending on the infecting \textit{C. neoformans} isolate, prompted us to investigate whether the IL-33 response could be induced in a similar manner. Interestingly both CBA and Balb/c mice show increased \textit{Il33} expression when infected with \textit{C. neoformans} H99 compared to infection with \textit{C. neoformans} 52D (Figure 11 and 12). It is also notable that Balb/c mice succumb to H99 infection
at 4 weeks (95) while CBA succumb at 7 weeks post-infection (173), and that \textit{Il33} expression is much higher in Balb/c mice compared to CBA. The inverse correlation between survival and \textit{Il33} mRNA expression levels suggest that \textit{Il33} may be a marker of susceptibility during \textit{C. neoformans} infection, although this possibility requires further study. Future experiments that compare the induction of T\textsubscript{h}1 and T\textsubscript{h}2 cytokines could also determine whether CBA mounts a more T\textsubscript{h}1 skewed response than that of Balb/c mice, as predicted by their respective \textit{Il33} expression levels.

Following the demonstration that IL-33 mRNA expression \textit{in vitro} and IL-33 protein and mRNA expression \textit{in vivo} is correlated with susceptibility to \textit{C. neoformans} infection, we wanted to determine what effect the absence of its signaling pathway would have on survival after infection. We obtained mice on the Balb/c background that were lacking the IL-33 receptor T1/ST2. And subsequently showed that mice with defective T1/ST2 dependent signaling have a significantly reduced lung fungal burden 3 weeks after infection with \textit{C. neoformans} H99 compared to wild type Balb/c mice (Figure 13A). These mice also had significantly reduced CFU in their spleens and brains as that compared to wild type controls 3 weeks after infection (Figure 13B and 13C). Interestingly, using a double knockout mouse lacking both IL-4 and IL-13, Zhang et al. were also unable to demonstrate differences in Brain CFU or survival following \textit{C. neoformans} H99 infection (95). These observations highlight the complexity of the host response to \textit{C. neoformans} and demonstrate that fungal dissemination from the lung is not simply mediated by the cytokines IL-4 and IL-13. Despite our currently limited availability of mice, we perform an initial survival study (data...
The preliminary results showed that the average survival of T1/ST2 knockout mice was 27 days compared to 24 days for the wild type controls; however, this trend is not statistically significant. Additional survival experiments and CFU counts at serial time points will be required to fully characterize the resistance of these mice.

**Proposed mechanism of susceptibility to *C. neoformans* via IL-33**

Given our data showing *Il33* expression by the epithelial lining cells of the airways and in a mouse model of *C. neoformans* infection, we are interested in characterizing the underlying pathway that causes susceptibility following the release of IL-33. IL-4–dependent IL-4 secretion from T_{H2} cells is a well-characterized pathway (178); however, the recent study of new innate cell populations such as nuocytes that produce large amounts of IL-13 in response to IL-33 signaling show that IL-4 responses can be potentiated through this mechanism (161). Interestingly, in a mouse model of *C. neoformans* infection, administration of an IL-4 neutralizing antibody reduced fungal burden and increased survival (179). Despite this manipulation, the level of IL-4 remained unchanged in the BAL, suggesting an IL-4–independent mechanism for activation of T_{H2} cells. Furthermore, another study showed that IL-13 knockout mice had similar survival and decreased lung fungal burden following *C. neoformans* infection (110). This group further demonstrated that wild type and transgenic animals for IL-13 had increased AAM while IL-13\(^{-/-}\) mice had increased CAM. They proposed that IL-13 was acting directly on the macrophages and inducing their phenotype; however, they did not show the cellular source of this cytokine.
As seen in a *Leishmania* model of infection, IL-13 has the potential to activate T\(_{H2}\) responses *in vivo* even in the absence of IL-4 expression (180,181). It is plausible that nuocytes activated by IL-33 produce large amounts of IL-13 that triggers AAM and causes them to harbor cryptococcal yeasts that are ultimately detrimental to the host. Recently, it has also been shown that IL-33 can be secreted directly from alveolar macrophages during influenza virus infection (160). To test this possibility in our model, exploratory experiments with peritoneal macrophages from Balb/c mice that were stimulated *in vitro* with *C. neoformans* H99 showed a trend towards higher *Il33* expression (*data not shown*). This finding suggests that macrophages may secrete IL-33 in response to *C. neoformans* infection that further potentiates an IL-13 mediated response via a positive feedback loop that involves innate lymphocytes.

Recently, a lineage negative nuocyte-type of cell population characterized by the expression of CD25, CD44, Thy1.2, ICOS, Sca-1 and IL-7α was shown to produce large amounts of IL-5 and IL-13 in the lungs of mice following administration of the fungal allergen *Alternaria alternate* (165). These cytokines led to the recruitment and activation of eosinophils without the need for T or B cells. We believe that this natural helper cell population could have a role during *C. neoformans* infection and could, at least in part, lead to the eosinophilia characteristic of susceptible *C. neoformans* models via the action of IL-5.

In summary, we propose the following model illustrated in *figure 14*. From our results we believe that IL-33 may either originate from the airway epithelial lining or may be released by alveolar macrophages following *C. neoformans* infection in susceptible mice that mount a T\(_{H2}\) response (*Figure 14*).
This IL-33 could then either activate an innate helper cell population, dendritic
cells, or T\textsubscript{H}2 cells directly, causing the release of IL-4, IL-5, IL-10 and IL-13. IL-
10 could act to repress T\textsubscript{H}1 cells and IL-4 and IL-13 could further potentiate the
T\textsubscript{H}2 response. IL-33, acting on innate helper cells via the receptor T1/ST2, could
cause the release of large amounts of IL-5 and IL-13. This IL-5 could act on
eosinophils, inducing the detrimental inflammatory response seen in susceptible
mice. Additionally the IL-13 could act to induce AAM that phagocytose and
harbor cryptococcal cells. Further detailed experimentation will be required to
elucidate the contributions of each of these pathways during \textit{C. neoformans}
infection.

**Future Directions**

With these initial observations for the role of IL-33 during \textit{C. neoformans}
infection, we must elucidate the underlying mechanisms for this phenomenon.
Three questions that remain to be answered following the work of this thesis
include: 1) How does the abrogation of IL-33 signaling confer resistance to
otherwise susceptible mice; 2) Could this effect be generalized to other epithelial
polarizing cytokines; 3) Can the abrogation of signaling by the Th1 cytokine IL-
1\beta confer susceptibility to \textit{C. neoformans} infection?

To answer the first question it is essential that we fully characterize the
inflammatory response in T1/ST2 deficient mice. In order to do this, enumerating
the CFU in the lung and other organs at 1, 2, 3 and 4 weeks will be essential to
understand the pattern of disease progression. Furthermore, histological analysis
may provide us with the initial clues to the specific cell types involved in this
response as the current literature seems to point to a plethora of targets for IL-33 
(Figure 5). Absent or diminished staining with periodic acid-Schiff (PAS) dye 
will provide a valuable clue if indeed these mice knockout have a reduction in 
airway mucus that is characteristic of a TH2 response. The total IgE concentration 
in mouse serum samples can be determined, as this has been shown to correlate 
with allergic responses and the susceptibility to C. neoformans infection (109). 
BAL analysis for airway cell recruitment and cytokine profile could also be 
performed, and FACS analysis could more precisely define the phenotype of 
specific cell populations in the BAL, lungs and lung-associated lymph nodes 
(LALN). Peritoneal macrophage experiments could be repeated using alveolar 
macrophages to confirm our previous observations. Finally, an experimental 
protocol using wild type mice has been established for the in vitro restimulation of 
CD4+ T cells from the LALN of mice previously infected with C. neoformans 
H99. These cells were then stained intracellularly for IL-4, IL-13, IL-17A and 
IFN-γ production and analyzed by flow cytometry. When more knockout mice 
become available, this protocol will help us elucidate the precise phenotype and 
contribution of CD4+ T cells to the host response against C. neoformans in the 
absence of T1/ST2 dependent signaling.

Initial evidence from our in vitro studies points to a role for IL-25 during 
C. neoformans stimulation; however, these results could not be reproduced in 
vivo. IL-25 expression was evident at an early time point relative to IL-33 
expression. Thus, it may be possible to obtain confirmatory results in vivo by 
analyzing a more appropriate time point following infection then was done in this 
work. The availability of IL-17RB knockout mice at McGill University could
provide a definitive answer regarding the possible role of IL-25 in host resistance to *C. neoformans* infection. If these studies implicate IL-25 in the immune response to *C. neoformans* H99, the analysis of double knockout mice for T1/ST2 and IL-17RB (breeding currently underway) could reveal a very interesting phenotype. Furthermore, the study of nuocytes, a unique innate lymphoid population that expresses both T1/ST2 and IL-17RB on the cell surface, may yield a new novel immune mechanism during *C. neoformans* infection. These cells have been previously characterized at McGill University using mice with a green fluorescent protein inserted into the *IL13* promoter region. Thus, many tools are at our disposal to fully elucidate both the cellular sources and cellular targets for epithelial polarizing cytokines during *C. neoformans* infection.

Finally, characterization of the role of \( T_{H1} \) cytokines such as IL-1\( \beta \) during *C. neoformans* infection could help to strengthen our proposed model (*Figure 14*). To date, we have infected susceptible C57BL/6 mice lacking the IL-1\( \beta \) receptor, IL-1R1, with *C. neoformans* 52D. At 5 weeks post-infection there was no difference in fungal burden, and these results were recently confirmed by another group (182). We believe that the role of IL-1R1 may be more evident in a resistant genetic background; therefore, IL-1R1 knockout mice on a Balb/c background are being generated through serial backcrossing. If these mice are susceptible to *C. neoformans* infection, it will add further evidence to the growing body of literature suggesting that allergic inflammation during *C. neoformans* infection is detrimental to the host.
Figure 14: Proposed model of IL-33 function during *C. neoformans* infection.

In susceptible hosts that mount a predominantly Th2 response, IL-33 is released from damaged epithelial cells and from lung resident macrophages (MΦ). IL-33 has a wide spectrum of action including activation of dendritic cells (DC), direct activation of T-helper type 2 cells (Th2), and activation of alternative macrophages (aaMΦ). Th2 cells further propagate this response by secreting IL-4, IL-5, IL-10, and IL-13 and cause class switching to IgE in B cells (B). IL-10 downregulates Th1 responses while IL-5 activates eosinophils and causes the eosinophilia associated with *C. neoformans* infection. Conversely, resistant hosts may mount a predominantly Th1/Th17 response characterized by the phagocytosis and clearance of *C. neoformans* by neutrophils (NT), MΦ, and DCs. DCs present antigen to naïve T cells and, through the action of IL-12, cause them to differentiate into Th1 cells. In turn, this leads to the secretion of IFN-γ that classically activates macrophages (caMΦ). Increased IL-1β expression has been observed in resistant mice, although the precise role of the IL-1β–IL-1R1 signaling axis during *C. neoformans* infection has not been thoroughly investigated.
CONCLUSION

With reports indicating that cryptococcal disease is now emerging in the immunocompetent population, it is particularly important that we delineate the molecular mechanisms of susceptibility and resistance that underlie infection with *Cryptococcus*. These studies were the first attempt to investigate the role of epithelial polarizing cytokines during *C. neoformans* infection. We found that IL-33-dependent signaling is a crucial mechanism of allergic (TH2) lung inflammation that confers susceptibility to progressive cryptococcal pneumonia, meningitis, and disseminated disease. Although the role for TSLP and IL-25 during *C. neoformans* infection still remains unclear, this current progress has provided the initial groundwork necessary for the further study of these cytokines during *C. neoformans* disease. With future studies we hope that the entire immune pathway initiated and potentiated by cytokine IL-33 in mice infected with *C. neoformans* can be elucidated with the hope that this will lead to novel or improved therapeutic targets for patients affected by *C. neoformans*. 
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