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LUNG EOSINOPHIL ATERATIONS IN A *CRYPTOCOCCUS NEOFORMANS* MURINE INFECTION MODEL

A Major Qualifying Project Report

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ABSTRACT

Cryptococcosis, infection with a fungus in the *Cryptococcus* genus, usually *neoformans*, is responsible for the deaths of hundreds of thousands of immunocompromised individuals worldwide annually. We hypothesized that the type of polarization of lung macrophage cells that form during infection of a murine model will provide evidence for the mechanisms of protection against this pathogen. We used flow cytometric analyses to assay specific marker proteins on macrophage cells recruited to the mouse lung during infection with various virulent *C. neoformans* strains. The results identified a previously unobserved factor that helps determine whether the mouse succumbs to infection, and the project shifted to examine distinct eosinophil populations recruited by the murine strains that were most susceptible to *Cryptococcus*. This information will hopefully aid in the Levitz's lab development of a vaccine against *C. neoformans*.

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BACKGROUND

Cryptococcus neoformans is a fungus that most people come into contact with daily. The presence of this opportunistic pathogen in the environment is usually innocuous for the majority of the population, as it typically does not affect immunocompetent individuals. First identified as a human pathogen in 1894, *Cryptococcus neoformans* is responsible for approximately 700,000 deaths worldwide annually, mostly affecting individuals with repressed immune systems (Casadevall and Perfect, 1998; CDC, 2016). The *Cryptococcus* problem persists; there is no available vaccine for the fungus, and current treatments have many side effects. While medical advances over the past century have helped prolong human life expectancy and the quality of life, the ability to knock down the immune system for some medical treatments has increased the pool of immunocompromised hosts in which *Cryptococcus* can reside.

Cryptococcus neoformans and Pathogenesis

Fungi classified as *Cryptococcus* are categorized as non-fermentative, urease-producing yeasts (Casadevall and Perfect, 1998). The fungus can be isolated from avian excreta, predominantly pigeon, and from some types of decaying wood (CDC, 2016). The fungal cells are protected by a distinguishing polysaccharide capsule whose production can be induced both in environmental and infected host settings (O'Meara and Alspaugh, 2012).

Infection with *C. neoformans* is termed cryptococcosis. Of the 38 identified species of *Cryptococcus*, reports of human pathogenicity are most often for species *neoformans* and *gatii* (Casadevall and Perfect, 1998). Infection can occur upon inhalation of fungal spores, but it cannot be passed from human to human (CDC, 2016). Yeast cells most often act as the infectious particle of the fungus; at 1.8-2.0 μm they are small enough to easily aerosolize and penetrate the lung alveoli (Rodrigues et al., 1999). The wide geographic range of *C. neoformans* results in regular exposure for most individuals, and immunocompetent people are asymptomatic (Merck Manuals, 2016). However, introduction of cryptococcal spores into the pulmonary system of immunocompromised individuals is often fatal, and results in 1 million cases of cryptococcal meningitis in T-cell-deficient individuals annually (CDC, 2016). If untreated, the pneumonia-like infection in the lung can disseminate and attack the central nervous system (CNS) (Steenburger and Casadevall, 2003).

Within the lungs, the fungal cell is phagocytosed by alveolar macrophages (**Figure-1**). This facultative intracellular pathogen then utilizes the phagocytes to spread throughout the body. Although macrophages normally produce oxidative and nitrosative agents to create a hostile environment that kills invading pathogens, *C. neoformans* sometimes survives intracellularly in macrophages, which helps disseminate the disease and provides resistance to antifungal agents. The crossing of infected macrophage cells across the blood brain barrier to infect the central nervous system is most often fatal. The mortality for AIDS infected individuals who contract cryptococcal meningitis is about 60% (Specht et al., 2015).

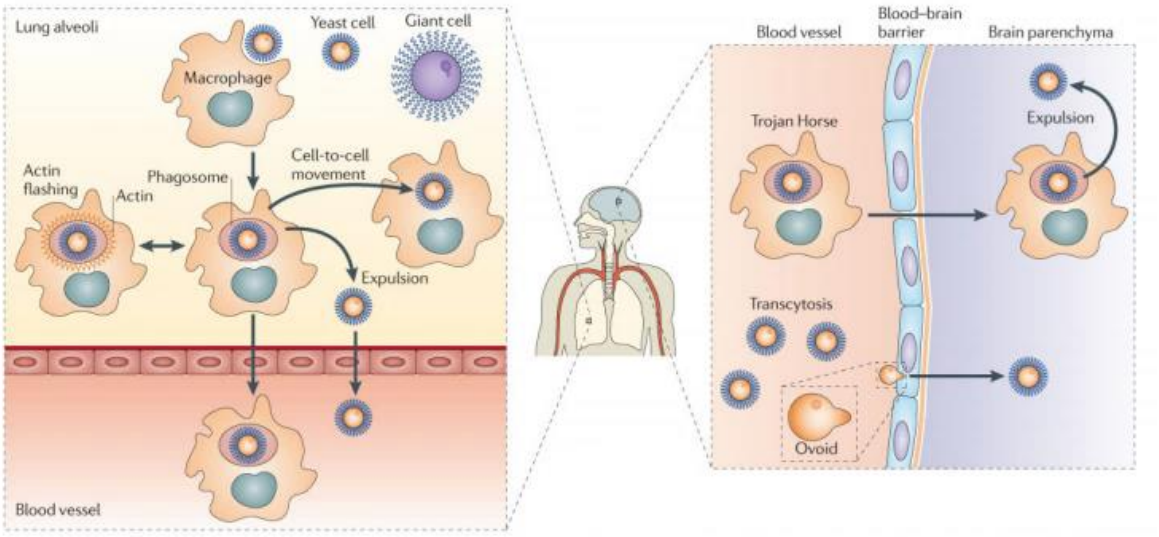


Figure-1: Diagram of the Uptake of *Cryptococcus neoformans* Spores by Lung Macrophages. Left Panel denotes the initial interaction of *C. neoformans* with alveolar phagocytic cells, its cell-to-cell movement, and the movement of infected macrophages into the circulation. **Right Panel** denotes the subsequent crossing of infected macrophage cells across the blood-brain barrier into the central nervous system (Kronstad et al., 2011).

One mechanism by which *C. neoformans* survives the hostile intracellular environment of the macrophage involves upregulation and secretion of fungal virulence factors (**Figure-2)**. These factors influence the macrophage intracellular environment, and the expulsion and transfer of the fungus between cells. The factors are delivered by membrane-bound extracellular vesicles that traverse the yeast cell wall. Thus, the Golgi/endosome/exocytosis pathway (diagram center) and membrane blebbing from the plasma membrane with captured cytoplasmic material (upper diagram) is thought to be important.

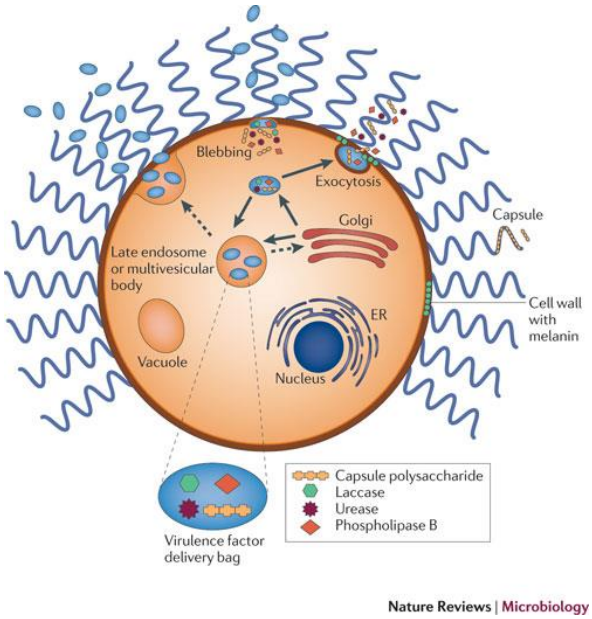


Figure-2: Schematic of *Cryptococcus neoformans* Pathogenesis. Shown are the secretion of virulence factors either by the Golgi-endosome-exocytosis pathway (diagram center), and the blebbing of the plasma membrane containing captured cytoplasmic material (upper diagram) (Kronsted et al., 2011)

The fungal virulence factors thought to be important in a *C. neoformans* infection (**Table-1**) include the synthesis and secretion of the polysaccharide capsule, the enzyme laccase (which synthesizes melanin), phospholipase-B, and urease (also shown as colored items in **Figure-1**). The defensive capsule is a major contributor to the virulence of the fungus. It is composed mainly of glucuro-noxylomannan which can inhibit phagocytosis (Rohatgi and Pirofski, 2015). It can also contain melanin which aids in the retention of fungal hydrolytic enzymes by binding their products and keeping them nearby and readily available to the fungus (Casadevall et al., 2000). The virulence factor delivery bags released into the bloodstream (blue in **Figure-2**) contain polysaccharides which decrease leukocyte migration to the site, phospholipases which are critical in host cell wall destruction,

urease which causes a spike in local pH, and laccase which protects against oxidative burst by alveolar macrophages (Steenbergen and Casadevall, 2003).

Table-1: Virulence factors of *Cryptococcus neoformans* and their functions in both the environment and pathogenesis (Casadevall et al., 2003)

'Dual use' virulence factors of *C. neoformans.**

	Function	
Attribute	In the environment	In pathogenesis
Capsule	Prevents dessication [16]	Antiphagocytic [28]
	Protection against amoeboid predators [12]	Immunomodulator [29]
		Intracellular agressin [6]
Laccase	Lignin degradation [30]	Interference with oxidative burst [31]
Melanin	Ultraviolet shielding [17]	Resistance to oxidative killing [32]
	Heat and cold tolerance [18]	Antiphagocytic [32]
	Reduced susceptibility to enzymatic degradation [33]	Immunomodulator [34]
	Protection against heavy metals [19]	Resistance to microbicidal peptides [20]
	Protection against amoeboid predators [12]	Antifungal-drug resistance [35]
Phospholipase	Nutritional function? [36]	Intracellular growth [37]
	Protection against amoeboid predators [12]	
Proteases	Nutritional function [38]	Tissue damage [39]
Urease	Nitrogen scavenging [40]	Intracellular growth [40]
Phenotypic switching	Generation of strain diversity to survive environmental stress?	Immune evasion [41,42]
Mating type	Sexual reproduction [43]	Virulence factor regulation [43]
Calcineurin and cAMP signalling	Development and reproduction [44,45]	Virulence factor regulation [44,45]
Superoxide dismutase	Protection against oxygen-derived oxidants?	Intracellular growth [46]

*Not a complete list of virulence factors

Potential Vaccines and Implications

Laboratories worldwide are attempting to manipulate the innate immune response to render an effective vaccine against virulent strains of *C. neoformans*. The vaccine would not only have to be effective in fighting an established fungal infection, but must also prevent recolonization (Datta and Pirofski, 2006). Currently, the treatment for cryptococcal infections is an antifungal regimen, but in HIV and organ transplant patients this can lead to *Cryptococcus*-related immune reconstitution inflammatory syndrome (IRIS) which is life threatening (Leopold Wagner and Wormley, 2015). However, there is much debate whether any vaccine would work in individuals who are already immunocompromised. The countless immune system interactions which both prevent and fight infection must be taken

into consideration while developing a vaccine model in immunocompromised individuals.

M1 and M2 Macrophage Responses

The recruitment of leukocytes, or white blood cells, in response to infection is critical in both innate and adaptive immunity (Murphy, 2012). Maturing in the bone marrow from pluripotent hematopoietic stem cells, some leukocytes reside in peripheral tissues while others are circulated throughout the lymphatic system (Murphy, 2012). **Figure-3** illustrates the types of cells composing the body's leukocyte population. Leukocytes can be classified as granular or agranular, all derived from a common stem cell lineage (Murphy, 2012). Granular leukocytes are phagocytes characterized by nuclei with multiple lobes and the presence of cytoplasmic granules (Serhan et al., 2010).

Macrophages are especially important to this MQP project. Macrophages are agranular leukocytes that are mature monocytes. Macrophages are present in virtually all tissues, and their two major functions in the immune system are to: 1) phagocytose invading pathogens, and 2) induce inflammation to signal an immune response from other cells (Murphy, 2012). Based upon the stimuli received by these phagocytes, they may be activated to polarize to one of two phenotypes: classically activated M1 macrophages stimulated by TH1 Helper cells, or alternatively activated M2 macrophages stimulated by TH2 Helper cells (Chávez-Galán et al., 2015; Gordon, 2003). The M1 phenotype is defined as macrophages that have been activated by IFN- ϒ, have high antigen-presenting capacity, and produce large amounts of toxic intermediates, especially iNOS (Montovani et al., 2004). Contrastingly, M2

macrophages are activated through IL3/IL4, have high levels of non-opsonic receptors, and predominantly utilize the arginase pathway (Gordon, 2003; Montovani et al., 2004).

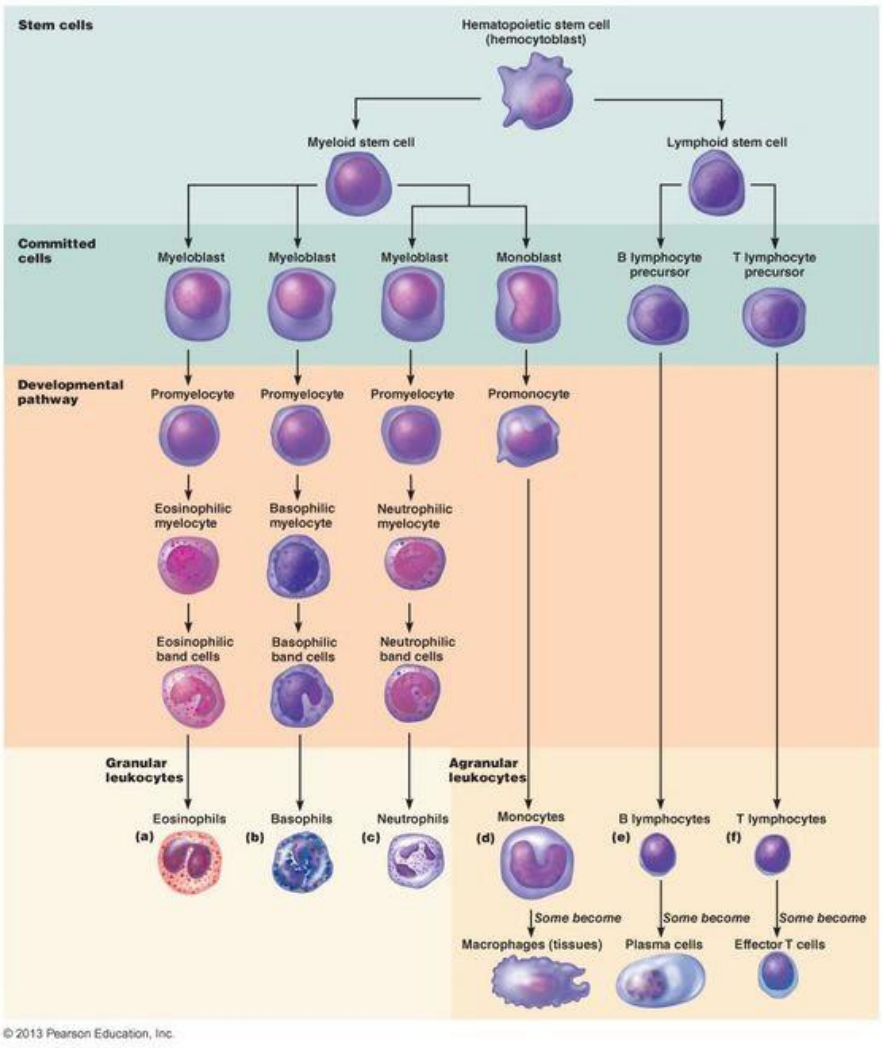


Figure-3: Diagram of Hematopoiesis. Shown are the main pathways of differentiation of hematopoietic stem cells to create the various cellular components of blood, including those of interest to this MQP such as: macrophages, B-lymphocytes and T-lymphocytes (diagram lower right). (Murphy, 2012)

Cryptococcus-Macrophage Interactions

Lung alveolar macrophages are the first line of defense for the immune system to inhaled pathogens (Kirby et al., 2009). After induction of *Cryptococcus* into the airway, the skewing of macrophages is a major factor determining whether an organism will succumb to infection. Classical macrophage activation and the shift towards M1 results in fungicidal cellular products, while unbridled fungal growth and exacerbation of disease results from a shift towards M2 (Leopold Wager and Wormley, 2015).

Figure-4 illustrates the effects of macrophage polarization on cryptococcal proliferation. The M2 phenotype (lower portion of the figure) is permissive to Cryptococcal growth, while induction of skewing to the favorable M1 phenotype (diagram upper) has the potential to aid in vaccine creation. While it has been experimentally demonstrated that M2 macrophages can be completely repolarized to M1 macrophages through changes in cytokine concentrations, other factors come into play *in vivo* that must be analyzed before effective therapeutic manipulation of the macrophage plasticity can be achieved (Davis et al., 2013).

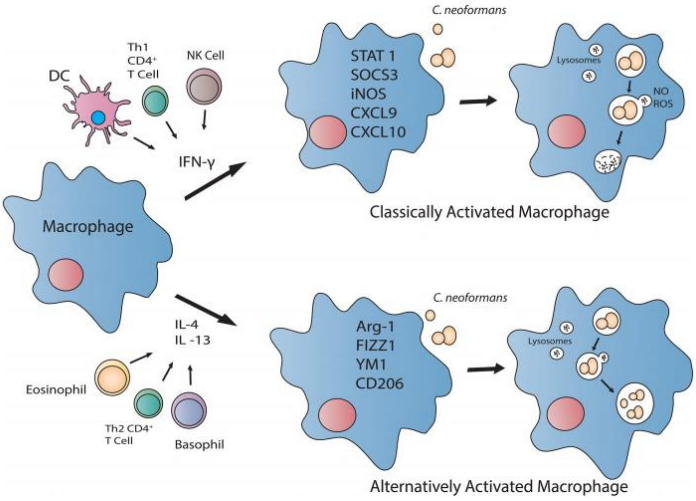


Figure-4: **Macrophage Polarization and Cryptococcal Infection**. Shown are the shift of lung macrophage cells to an M1 type (lower diagram) or an M1 type (upper diagram). The latter is a more effective response for fighting a Cryptococcal infection. (Leopold-Wager and Wormly, 2015).

Experimental Models and Infections in the Levitz Laboratory at UMMS

Mice are regularly used as a model organism, and in the Levitz laboratory at the University of Massachusetts Medical School, murine models are used to examine the effects of cryptococcal infection. The three murine strains most commonly used in this lab and their wild-type blood leukocyte compositions are shown in **Table-2**.

Table-2: Leukocyte Composition of Various Murine Models in our Lab (Jackson Labs, 2016)

Murine Strain		Percent Lymphocytes	Percent Basophils	Percent Monocytes	Percent Neutrophils	Percent Eosinophils
Balb/C	Male	71.9	0.8	0.9	23.1	2.9
	Female	80.6	0.2	0.8	14.8	3.0
CBA/J	Male	76.9	0.3	1.4	17.7	3.3
	Female	79.9	0.2	1.5	15.8	2.2
C57BL/6	Male	76.7	0.2	1.4	18.5	2.6
	Female	88.6	0.2	0.7	7.4	2.6

As key players in the immune response, the leukocyte cells listed in the table above are determining factors in whether a murine model will succumb to infection. The Levitz laboratory works most often with two strains of *Cryptococcus*: Kn99α (kn99) and cda1Δ2Δ3Δ (cda123). Kn99 is a hyper-virulent strain of the fungus which was backcrossed ten times with the clinical strain Hn99. Cda123 is an attenuated mutant strain of *C. neoformans* derived from kn99 which lacks cell wall chitosan (Specht et al., 2015). Infections with the same strain of *Cryptococcus* in different murine lines result in varying clearance of the fungal load. **Figure-5** illustrates the clearance of a cda123 *neoformans* infection in C57BL/6 and CBA/J mice (Lee et al., unpublished data). Attenuated cda123 is cleared from the lungs by day 7 post-infection in both mouse strains, while in contrast, most murine stains are more susceptible to virulent kn99 (data not shown).

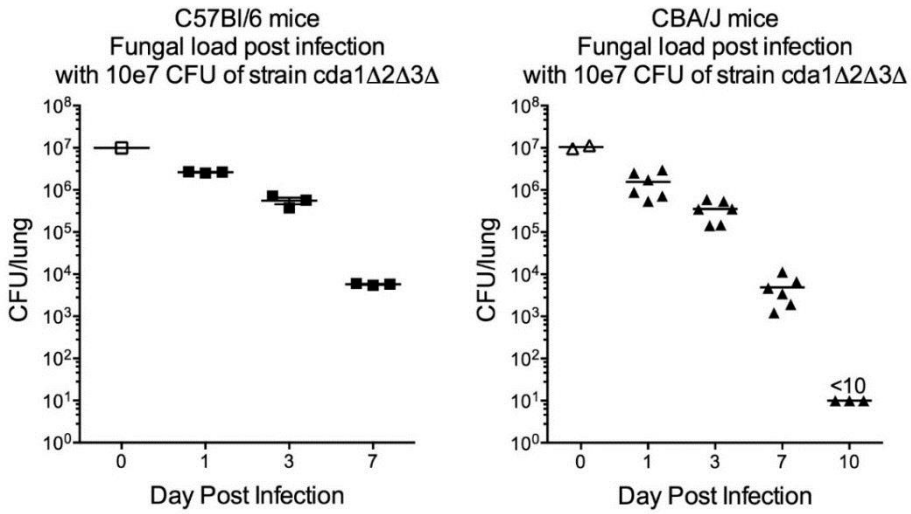


Figure-5: Infection of two Mouse Strains with C. neoformans. Shown are the plots of colony forming units of C. neoformans in C57/BL/6 mice (left panel) and CBA/J mice (right panel) (Upadhya, unpublished data).

Figure-6 illustrates the test of a vaccination approach using heat killed *C. neoformans* (HK in the diagram). The figure shows the survival of 4 strains of mice infected with heat-killed cda123 as a trial vaccine, and then subsequently challenged with a second infection 40 days later. All unvaccinated mice die around 20 days post infection, while the vaccinated A/J, BALB/c and 129 mice show a high rate of survival. The vaccinated C57/BL/6 mice die by day-60. FACS analysis of leukocyte composition revealed that the C57/BL/6 mice have a substantial eosinophil population which appears to negatively correlate with a model's ability to recover from infection.

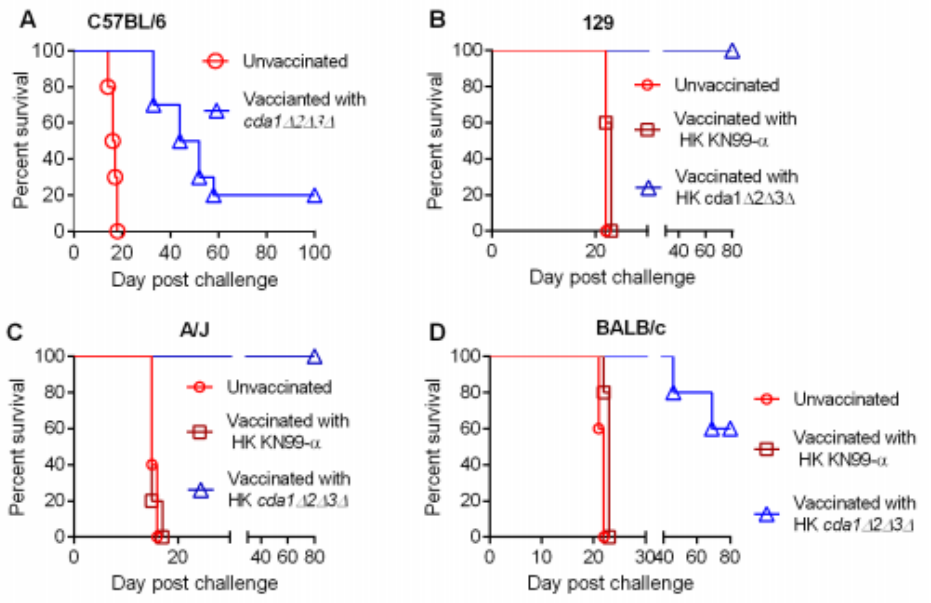


Figure-6: Test of a Vaccination Approach Using Heat-Killed *C. neoformans*. Survivorship of murine strains vaccinated with heat-killed cda123 and then challenged with a cryptococcal strain 40 days later (Upadhya, unpublished data).

PROJECT PURPOSE

Cryptococcus neoformans infections are opportunistic in immuno-compromised individuals, and are increasing as a greater portion of the population become immunocompromised from the use of immunosuppressive drugs or from AIDS. The long term goal of the Levitz Lab at UMMS is to develop a vaccine which will protect against cryptococcal infection in immunocompromised individuals. *C. neoformans* infects the lungs, and is taken into macrophage cells. Depending on the hormones present and other factors, the infected macrophages can either develop down an M1 pathway (desirable for vaccines), or down a M2 pathway. Different mouse strains (containing different populations of leukocytes) respond differently to *C. neoformans* infection. The objective of this MQP project was to characterize lung macrophages in murine models of post-oral-tracheal injection with *Cryptococcus*, to help uncover the mechanism by which different murine lines become protected against virulent strains of the fungus. The hope was to obtain information for eventual therapeutic manipulation of the pathway.

The initial investigation focused on the use of FACS to monitor the M1 and M2 immune responses. However, it became evident there was a previously unobserved factor that determines whether the mouse model succumbs to infection, and the project shifted to examine distinct eosinophil populations recruited by the murine strains most susceptible to *Cryptococcus*. This information will hopefully aid in the Levitz's lab development of a vaccine against the fungus.

METHODS

Infection of Mice with *C. neoformans*

Two strains of *Cryptococcus neoformans* were used throughout the course of these experiments: attenuated cda123 and virulent kn99. Mouse strains Balb/C, C57Bl/6, and CBA/J from Jackson Laboratories were used as murine models. Mice were infected oral-tracheally with 10^6 CFU of cda123 and 10^4 CFU of virulent Kn99. Mice were euthanized for removal of the lungs to be studied through the procedures described below.

Lung Dissociation

After dissection of murine lungs into single lobes, samples were physically and enzymatically dissociated using a Miltenyi Biotec Lung Dissociation Kit. Lungs were added to a gentleMACS C Tube containing 2.4 mL 1X Buffer S, 100 µL Enzyme D, and 15 µL Enzyme A. The C Tubes were then attached upside down to the gentleMACS Dissociator and run on program m_lung_01 to begin the mechanical dissociation. Next the tubes were incubated for 30 minutes at 37°C on the MACSmix Tube Rotator for continuous rotation to promote enzymatic degradation of the extracellular matrix. After this incubation, the tubes were again placed upside down on the gentleMACS Dissociator, and they were run on program m_lung_02 for further mechanical dissociation. The resulting suspension was passed through a 70 µm filter to remove any remnants of large particles.

200 µL of the suspension was transferred to a plate for determination of colony forming units (CFU) to validate that the fungal infection took. 200 µL of each solution was placed in a 1.5 mL Eppendorf tube. For kn99 samples the suspension was diluted 1:10 with 1X PBS. 15 µL of each dilution from 10^{-1} to 10^{-6} were plated. For cda123 infected samples, 150 µL of undiluted suspension were dispensed onto the plate and spread using a bent pipette tip. Plates were incubated at 37°C for 48 hours before colony counts were obtained.

Lung Leukocyte Extraction

The cell suspension created with the Lung Dissociation Kit was centrifuged for 5 minutes at 1500 rpm to pellet to begin the leukocyte extraction. After discarding the supernatant, the pellet was resuspended in 5 mL of 40% Percol (GE Healthcare) in a 15 mL conical tube. The lung cell and 40% Percol mixture was then slowly and carefully layered onto 5 mL of 66.67% Percol solution in a 15 mL conical tube. The tube was next centrifuged at 2000 rpm for 20 minutes with no brake to create a Percol gradient wherein the leukocytes became isolated at the interface between the 40% Percol and 66.7% Percol. After centrifugation, the top layer of *Cryptococcus* and the 40% Percol solution were pipetted out of the tube and discarded. The leukocyte layer was removed with a P1000 and placed in a new 15 mL conical tube to be washed with 10 mL of 1X PBS. This mixture was centrifuged at 2000 rpm for 5 minutes to pellet and remove any remaining Percol before being resuspended in 1X PBS.

Cell Staining

A cell count was next obtained for the extracted leukocytes. 10 µL of the cell suspension was pipetted into a hemocytometer and counted at 100X magnification under the compound microscope. Cell counts were later used to determine volume of suspension necessary depending on the type of staining to be done.

After an additional wash in 1X PBS, the pellet was suspended in 100 µL of FACS Buffer and transferred to a 1.5 mL Eppendorf tube. To block FC receptors, 1 µL of .5 mg/mL FC-Block was added to the tube. The sample was incubated on ice for 20 minutes before two washes with FACS buffer.

For cell surface staining, the pellet was resuspended in 100 µL of FACS buffer. 1 µL of each selected antibody (**Table-3**) was then added to the tube.

Table-3: Antibodies used for Cell Surface Staining

Antibody	Company
CD45	BD Bioscience
CD11b	eBioscience
CD11c	eBioscience
Ly6G	Bio Legend
Siglec-F	BD Bioscience

After the addition of the antibodies selected from the above table, the sample was incubated on ice for 30 minutes. For samples subject only to surface staining, the cells were then washed twice more with FACS buffer. The final pellet was resuspended in 300 µL of FACS buffer and fixed with an additional 300 µL of 4%

paraformaldehyde (Electron Microscopy Sciences) in 1X PBS. Samples were stored at 4°C until scheduled FACS analysis.

FACS Analysis

The samples created were analyzed by flow cytometry. The channels for the various fluorophores used over the course of experiments are shown in **Table-4**.

Table-4: Antibodies and their Fluophores

Antibody	Fluorophore
CD45	APC-Cy7
CD11b	Pe-Cy7
CD11c	Pe
Ly6G	PerCP-Cy5.5
Siglec-F	Pacific Blue

In addition to the channels selected for specific samples, side scatter, forward scatter, forward scatter area, and forward scatter height were also measured by the machine. Single color controls created using OneComp eBeads (eBioscience) were run during every FACS session to be used for compensation in further analysis. The raw FACS data was analyzed using FlowJo 5.6.7 software.

RESULTS

Cases of cryptococcosis, infection with *Cryptococcus neoformans*, have been on the rise as the host range for this opportunistic pathogen increases with the population of immunocompromised individuals. In addition to the HIV/AIDS epidemic which began in the 1980s, medicine has evolved in such a way that the immune system can be suppressed for certain therapies, such as bone marrow transplants; so illness is no longer the only way in which the immune system can be repressed, and the opportunity for cryptococcal infection has expanded. In attempts to gain insight into mechanisms of protection for vaccine development against the fungus, cells recruited to the lungs of murine models during infection with either cda123 or Kn99 were observed through FACS analysis.

After extraction of leukocytes from the enzymatically and mechanically digested murine lungs, the cells were stained using antibodies against CD11b, CD11c, CD45, Ly6G, and Siglec-F, on either day 3 or day 7 post-infection. After flow cytometric analysis, the raw data was analyzed by FlowJo5.6.7 software. Compensation using single color controls was adjusted before selecting for singlets and gating out debris (**Figure-7**).

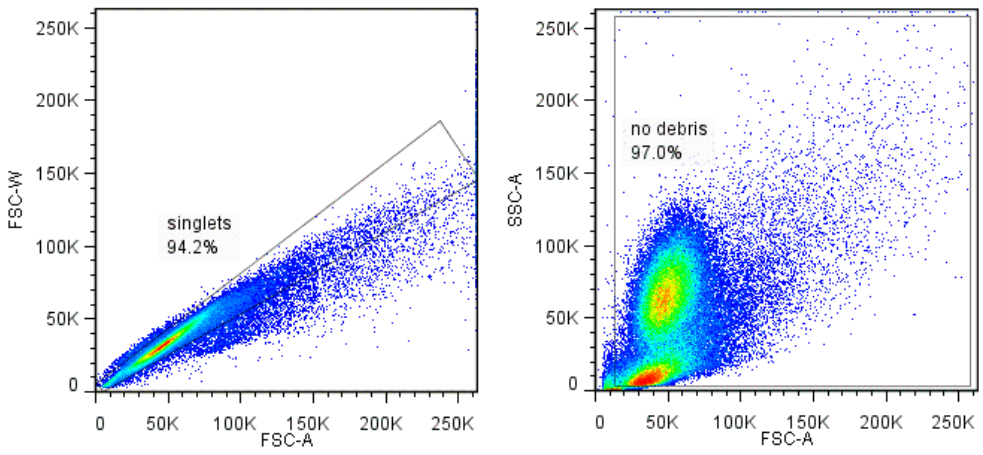


Figure-7: Initial FACS Gating Steps. The first steps in the FlowJo5.6.7 software were to select for singlets and gate out debris. Singlet cells were selected by graphing forward scatter area (X-axis) against forward scatter height (Y-axis) (**left panel**). Cell debris was removed by graphing forward scatter (X-axis) against side scatter (Y-axis) (**right panel**). In this plot, cell debris is denoted by a high Y-axis placement, which is minimal in the data shown.

When these initial steps in the gating process were applied to Kn99 (virulent) or CDA123 (attenuated) infection of CBA/J, BALB/C, or C57BL/6 mice (**Figure-8**), the FACS analysis revealed a distinct population of high side-scatter cells (circled black in the diagram) (granular lymphocytes) that were especially predominant in Kn99-infected C57BL/6 mice. The population was more visible at day 7 than day 3, was not observed in the more resistant CBA/J mice, and was intermediate in the Balb/C strain (**Figure-8**).

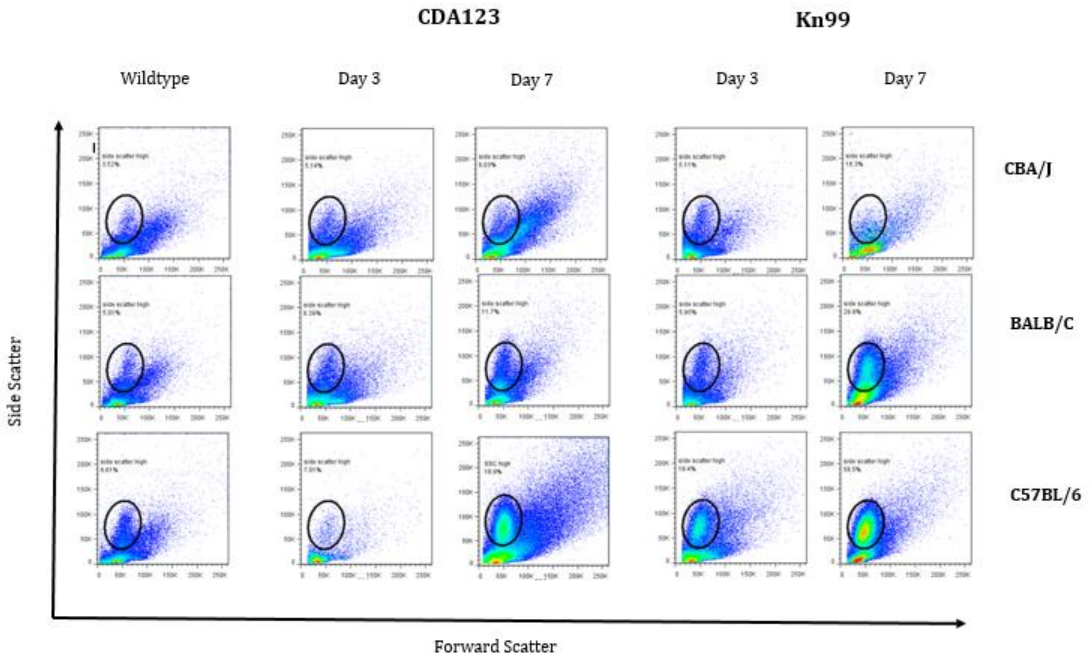


Figure-8: Presence of High Side Scatter Cell Populations in *C. neoformans* Infected Mice. After selecting for singlet cells, and gating out cell debris, a population of high side-scatter cells was observed (circled black in the panels). The population was best seen in Kn99-infected C57BL/6 mice 7 days post infection, and was minimal in CBA/J mice.

The plating of lung homogenates for assaying fungal colony forming units (CFU) (**Figure-9**) showed that mice with a larger fungal Kn99 CFU load (beige histobars) also harbored a greater high side-scatter cells in their lung leukocytes (blue histobars). The BL/6 mice who have the highest percentage of stained leukocytes as high scatter also had the greatest load of Kn99 in the lung. Thus, the presence of these high side scatter cells appears to have a positive effect on fungal growth.

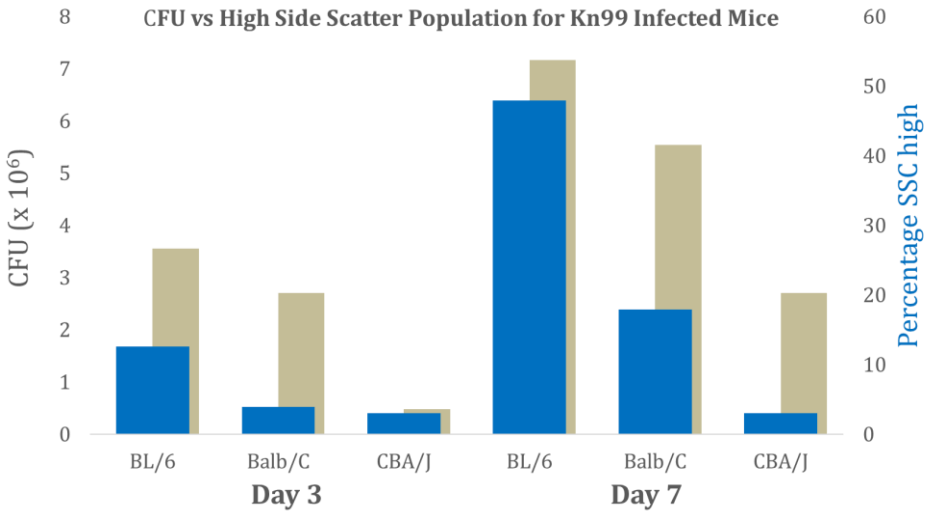


Figure-9: Correlation of *C. neoformans* Lung Colony Forming Units Compared to High Side-Scatter Populations. Fungal colony forming units of *C. neoformans* Kn99 (beige histobars) increase as the percentage of extracted leukocytes with high side scatter (blue histobars) increase.

Further FACS analysis was conducted with specific surface markers to determine the cell types present in the high side-scatter population (**Figure-10**). Additional gating was done to select for CD45+ cells, and to gate out B and T cells.

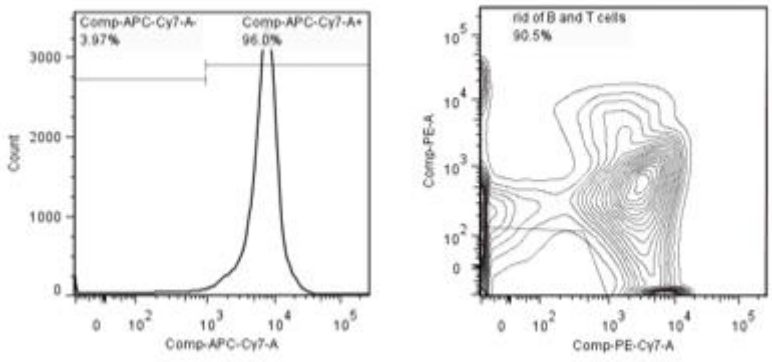


Figure-10: Final Gating Steps for Additional Analysis of the High Side Scatter Population. CD45+ cells (pan leukocyte marker) were selected for (**left panel**). Cells which were double negative for CD11b and CD11c were gated out to remove B and T cells from the analysis (**right panel**).

The additional gating steps were then applied to *C. neoformans*-infected mice (**Figure-11**) for cells stained for Siglec-F (strongly expressed on eosinophils and alveolar macrophages) against cells which fluoresced for CD11c (found at high levels on dendritic cells, but also on monocytes, macrophages, neutrophils, and some B-cells). The Kn99 infected BL/6 mice (susceptible) (left panel) showed a very strong population of cells that were high in Siglec-F and low for CD11c (Siglec-F^+, $CD11c^-$). Due to the high granularity of the cells, it was hypothesized that these cells may be eosinophils (Piehler et al., 2011).

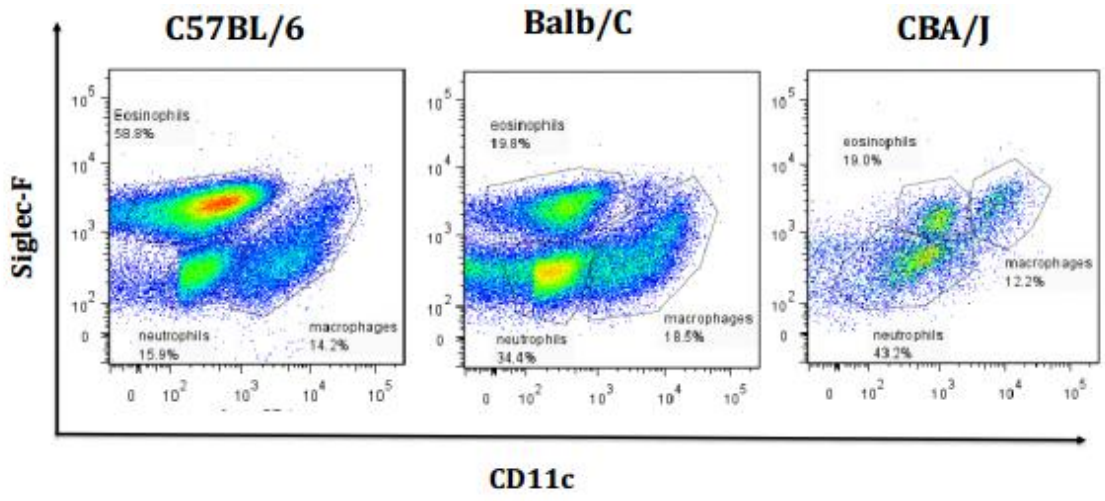
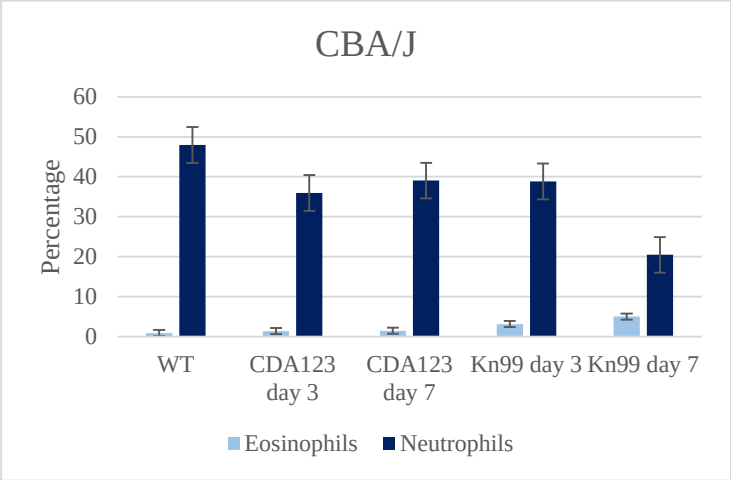
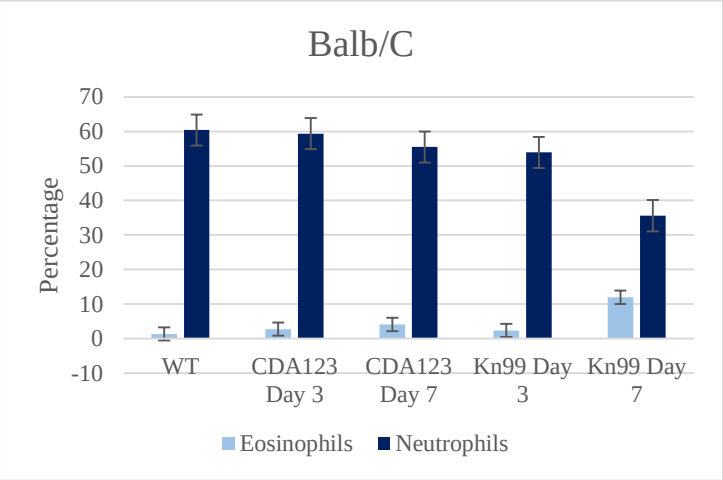


Figure-11: FACS Analysis of Leukocyte Populations in Lung Homogenates of Kn99 Infected Mice Day-7 Post-Infection. Eosinophil, neutrophil, and macrophage populations present in the lungs of infected mice were visible. Susceptible BL/6 mice (left panel) showed the highest level of eosinophils (Siglec-F+, CD11c-) (upper left circle). Percentages are not from the total cells analyzed but the percentage of the cells within the gate forming that population.

To determine the reproducibility of these findings, the FACS assays were repeated for both Kn99 and CDA123-infected mice (**Figure-12**). Thus, the mice who recruit a larger eosinophil and smaller neutrophil population to the lungs during infection,

such as the C57BL/6 mice, are more likely to succumb to infection than mice who lack this phenotype, such as the CBA/J mice.





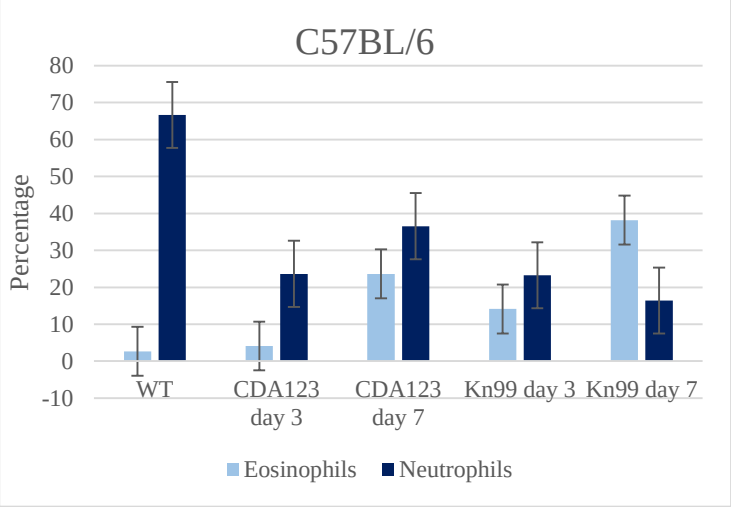


Figure-12: Summary of Repeated FACS Analyses of *C. neoformans* -Infected Mice. Shown are the estimates of eosinophil (light blue) and neutrophil (dark blue) cells at day-3 and day-7 post-infection with attenuated CDA123 and virulent Tn99 spores. Percentages of populations are based off of the total analyzed leukocytes, and are each the average of two experiments (N=2). The most drastic changes over time can be seen in the C57BL/6 mice (**bottom left**), whose eosinophil population surpasses the neutrophil population on Day 7 of a Kn99 infection.

DISCUSSION

The initial attempts to analyze lung cell populations in *Cryptococcus* infected murine models for M1 or M2 lung macrophage polarization did not result in clear data perhaps due to the plasticity of the macrophages, so the project took a different turn to investigate a population of high side-scatter cells (granular leukocytes) found to be present in BL/6 mice most likely to succumb to an infection. FACS analysis with Siglec-F and CD11c identified the population of cells as most likely being eosinophils, granulocytes known to be Siglec-F+, CD11c-. The high presence of these cells in Tn99-infected mice correlated with a higher fungal load in the lung. It appears the recruitment of this population of cells is detrimental to survival of the infected organism.

C57BL/6 mice exhibit a distinct high side scatter population when infected with Kn99 (**Figure-8**). This population is not present in the more resistant CBA/J mice and Balb/C mice that appear to exhibit an intermediate phenotype. While it is evident that certain murine strains are more susceptible to infections, the exact mechanism responsible remains unknown. The C57BL/6 mice with the high side scatter population, happen to be one of the strains more susceptible to *Cryptococcus* infection. In support of this, the percentage of total cells found in this high side scatter population corresponds with the mice harboring the greatest fungal loads of virulent Kn99 as assayed by CFU (**Figure-9**). The relationship indicates these high side scatter cells are not useful in clearance of the infection.

Staining of cells for surface markers Siglec-F and CD11c revealed eosinophil and neutrophil populations within the group of high side scatter (granulocyte) cells.

Both eosinophils and neutrophils are granular leukocytes. As the total eosinophil population in the lung increases, the total neutrophil population tended to decrease in mice infected with Kn99 (**Figure-12**). As the infection progresses, i.e. from day 3 to day 7, the eosinophil count increases. The most drastic shift was seen in the C57BL/6 mice where the percentage of eosinophils surpassed the percentage of neutrophils by day 7. Thus, maintaining an appropriate eosinophil-neutrophil balance seems to be an important aspect in the effectiveness of the immune response to Kn99.

Mouse models exhibiting a greater high side scatter (granulocyte) population also showed a higher eosinophil population, the greatest population being present in the day-7 Kn99-infected C57BL/6 mice (**Figure-12**). The reason for the decreased resistance of the BL/6 mice is unknown, but the strong presence of the eosinophils could be part of the driving force behind the susceptibility. Eosinophils secrete IL-4 (Davoine and Lacy, 2014). IL-4 is a cytokine used by our lab and others to skew bone marrow-derived cells to an undesirable M2 phenotype. IL-4 and Th2 cells drive alternate activation, and even in the presence of M1-promoting IFN-ϒ this cytokine is harmful to an organism with *Cryptococcal* infection (Piehler et al., 2011). So, the strong presence of eosinophils in susceptible mice could be promoting an M2 phenotype, and in turn facilitating fungal growth.

To further investigate the response to infection, several experiments could be conducted in the future. First, populations of cells could be isolated using the FACS sorter, and stained to validate the hypothesized cell types. A survivorship study for each of the mouse strains would be helpful in linking the cell types recruited to the lung with the ability to fight cryptoccocal infection. Infection

studies in eosinophil knockout mice could also be done to provide information into the role of the eosinophil population by viewing effects of their removal. Additionally, a Luminex panel quantitating which cytokine proteins are present in the supernatant of infected lung homogenates would offer a comprehensive view into the biochemical differences between murine strains. The compilation of all this data will offer insight into the mechanics of infection and subsequent immune responses, and hopefully aid the development of a vaccine against *Cryptococcus neoformans*.

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