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Anti-Cryptococcal Signaling in NK Cells

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Anti-Cryptococcal Signaling in NK Cells

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Abstract:

Natural killer (NK) cells are cytotoxic lymphocytes that target tumor cells, virally infected cells, and microbes. The anti-tumor and anti-viral components of NK cells have been extensively studied, however much less is known about their anti-microbial activity. In this thesis I investigate NK cell anti-microbial signaling against the yeast pathogen *Cryptococcus neoformans*. Previous studies found that *C. neoformans* activates a SFK→ PI3K→ Erk cytotoxicity pathway. In order to expand our understanding I explored the role of Rac1, which is commonly an intermediate molecule linking PI3K and Erk. Surprisingly, I found that in cryptococcal killing Rac1 acts as an activator of PI3K. Additionally, I found that Rac1 and SFK were both necessary, but independent in activating PI3K.

Additionally, I explored adhesion molecules that are involved in NK cytotoxicity. I found that beta-1 integrins are needed for cryptococcal killing. Beta-1 integrins were found to stimulate an ILK→ Rac1 pathway. This pathway is independent of the NKp30 receptor, that is also required for cryptococcal killing. Although beta-1 integrins function as adhesion receptors in tumor killing, loss of function studies showed that beta-1 integrins were not required for NK cells to adhere to *C. neoformans*.

The lack of integrin involvement in adhesion caused me to investigate the immune synapse between *C. neoformans* and NK cells. I found that the adhesion force was

lower compared to 721.221 tumor targets and NK cells. The adhesion force in tumor killing is dependent on actin remodeling proteins - Arp2/3 and formins. Interestingly cryptococcal killing required formins but not Arp2/3. Actin remodeling in a cryptococcal synapse was lower compared to a tumor synapse. However, inhibition of Arp2/3 reduced the level of actin remodeling in a tumor synapse to what is seen in a cryptococcal synapse.

Together, my data highlights the similarities and differences in NK cell mediated microbial killing compared to tumor killing. It reveals beta-1 integrins as possible anti-microbial receptors, a non-canonical role for Rac1 in cytotoxicity, and identifies the reduction in actin remodeling at the cryptococcal synapse as an explanation for the difference in kinetics between anti-fungal and anti-tumor killing.

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List of Abbreviations:

ADCC - Antibody-dependent Cell-mediated Cytotoxicity
AFM - Atomic Force Microscopy
AIDS - Acquired Immunodeficiency Syndrome
AM - Alveolar Macrophage
Arp2/3 - Actin-related Protein-2/3
ARPC - Actin-related Protein Complex
BLT - Benzyloxycarbonyl-L-lysine Thiobenzyl Ester
Btk - Bruton's Tyrosine Kinase
C3 - Complement Component 3
Cas - Crk-associated Substrate
CFU - Colony Forming Unit
CH - Calponin Homology
CLR - C-type Lectin Receptors
CR3 - Complement Receptor 3
CSF - Cerebrospinal Fluid
cSMAC - Central Supramolecular Activation Complex
CT - Computed tomography
DAP12 - DNAX activation protein of 12kDa
DC - Dendritic Cells
DNA - Deoxynucleic Acid
Erk - Extracellular Signal-regulated Kinases
ERM - Ezrin, Radixin And Moesin
FAK - Focal Adhesion Kinase
FH - Formin Homology
FHOD1 - Formin Homology 2 Domain Containing 1
FMNL1 - Formin Like-1
GalXM - Galactoxylomannan
GEF - Guanine Nucleotide Exchange Factor
GXM - Glucuronoxylomannan
HEK293 - Human Embryonic Kidney Cells 293
HIV - Human Immunodeficiency Virus
HLA - Human Leukocyte Antigen
HUVEC - Human Umbilical Vein Endothelial Cells
IFN-γ - Interferon gamma
ILK - Integrin Linked Kinase
iNOS - Inducible Nitric Oxide Synthase
IRAK4 - Interleukin-1 Receptor-associated Kinase 4 Protein
IS - Immune Synapse

ITAM - Immunoreceptor Tyrosine-based Activation Motif
ITIM - Immunoreceptor Tyrosine-based Inhibitory Motifs
L-DOPA - L-3,4-dihydroxyphenylalanine
LFA-1 - Lymphocyte function-associated antigen 1
MAPK - Mitogen-activated Protein Kinase
MBCD - β-methylcyclo Dextran
MCP-1 - Monocyte chemotactic protein 1
MHC - Major Histocompatibility Complex
MIP - Macrophage Inflammatory Protein
Mpr1 - metalloprotease 1
MR - Mannose Receptor
MTOC - Microtubule-organizing Center
NCRs - Natural Cytotoxicity Receptors
NETs - Neutrophil Extracellular Traps
NK - Natural Killer Cells
NLR - Nod-like Receptors
NLRP - Nod-like Receptor Protein
NO - Nitric Oxide
PAMPR - Pathogen Associated Molecular Pattern Receptor
PBMCs - Peripheral Blood Mononuclear Cells
PI3K - Phosphoinositide 3-kinase
pSMAC - Peripheral Supramolecular Activation Complex
SCID - Severe Combined Immunodeficiency
SHP - Src Homology 2 Domain Phosphatase
siRNA - Small Interfering RNA
SFK - Src Family Kinase
SHP - Src Homology 2 Domain Phosphatase
siRNA - Small Interfering RNA
sRBC - Sheep Red Blood Cells
STAT1 - Signal Transducer And Activator Of Transcription 1
TLR - Toll-like Receptors
TNF-α - Tumor Necrosis Factor Alpha
VCAM - Vascular Adhesion Molecule
VLA - Very Late Antigen
WASP - Wiskott–Aldrich Syndrome Protein

Chapter 1: Introduction

Project overview

Cryptococcosis is an infection by the encapsulated yeast pathogen *Cryptococcus.* Host defense against cryptococcosis is poorly understood. Although specific immune cells have been identified to combat the organism, the signaling pathways that are activated in these cells are not fully understood. This is especially true for natural killer (NK) cells, since they are studied more for their anti-tumor and antiviral capabilities. Current therapies against cryptococcal infection involves amphotericin B and fluconazole. These medications have severe side effects, such as amphotericin B causing nephrotoxicity, and both therapies can promote the development of fungal drug resistance. Even with appropriate anti-fungal treatment, patients with cryptococcal meningitis still have a 10 week mortality rate of 12% [1]. Anti-fungal therapies are also ineffective in patients with immune deficiencies - which is a common scenario in *C. neoformans* infections. Another strategy to combat *C. neoformans* is to enhance the host anti-fungal response through augmentative immunotherapy. NK cells are activated by *Cryptococcus* and mount a direct contact-mediated cytotoxic response as well as promoting a cytokine-mediated protective Th1 response [2]. Therefore, they are a prime candidate for anti-cryptococcal immune therapy. Current immunotherapy strategies involves the addition of Th1 cytokines (tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), IL-12 and IL-18) [3–6] or anti-cryptococcal antibodies [7]. However only a few of these therapies target NK cell function - such as IL-12 [8]. In order to design more specific and effective therapies a greater understanding of the signaling that occurs in NK cells after

cryptococcal stimulation is required. Investigating signaling will also help identify the presence of anti-cryptococcal receptors. Currently, only NKp30 has been identified as an anti-cryptococcal receptor on NK cells [9]. Since a mature immune synapse requires both activating receptors - such as NKp30 - and adhesion molecules [10], there is a likely possibility that additional anti-cryptococcal receptors are present on NK cells.

Cryptococcus

Overview

Cryptococcus is a genus under the kingdom fungi. *Cryptococcus* is made up of multiple species, some of which are pathogenic and cause infections worldwide. These pathogenic species infect both immunocompromised and immunocompetent individuals, and even with best available anti-fungal therapy patients who develop cryptococcal meningitis suffer a 10-week mortality rate of 9-12% [11]. Additionally, in human immunodeficiency virus (HIV) endemic areas cryptococcal infections pose a large health risk.

First described in 1894, *Cryptococcus* is part of the phylum *basidiomycota*, characterized by their ability to form basidium on which spores are grown [12]. *Cryptococcus* is part of the class *Tremellomycetes*. However, the genus *Cryptococcus* is polyphyletic, and span the orders *Tremellales*, *Trichosporonales*, *Filobasidiales*, and *Cystofilobasidiales* [13,14]. *C. neoformans*, and its close relative *C. gattii* belong in the family *Tremellaceae*. This placement was the result of ribosomal deoxynucleic acid

(DNA) matching, since *C. neoformans* does not have the characteristic globulus basidium present in other genuses inside the *Tremellaceae* family [15,16].

The *Cryptococcus* genus consists of 37 different species. Many of these species have been associated with humans disease, including *C. adeliensis, C. albidus, C. chernovii, C. curvatus, C. diffluens, C. gattii, C. humicola, C. laurenti, C. luteolus, C. macerans, C. magnus, C. neoformans, C. terreus,* and *C. unigutulatus* [17–31]. However, *C. neoformans,* and *C. gattii* cause the majority of cryptococcal disease in humans [32]. Despite evolution outside of humans, *Cryptococcus* has developed virulence mechanisms that allows evasion of the human immune system. Current hypotheses suggest that cryptococcal adaptations to evade amoebas could have also selected for organisms that could evade human macrophages [33]. When *C. neoformans* mutants were co-cultured with *Acanthamoeba castellanii*, mutations in virulence factors that provided protection against macrophages also provided protection against *A. castellanii [33]*. Survival in *A. castellanii* is associated with increased virulence factors - such as capsule, melanin, and phospholipase secretion [33]. Growth of *Cryptococcus* in another amoeba - *Dictyostelium discoideum* - enhanced the virulence of the *Cryptococcus* in mice [34]. This suggests that amoeba can alter the ability of *Cryptococcus* to colonize animal hosts. The presence of cryptococcal virulence factors and its ability to survive at 37°C explain the ability of *C. neoformans* to infect human, while other members of the *Tremellomycettes* class do not.

Virulence factors

It should be noted that cryptococcal virulence factors are focused on evading, monocytes/macrophages, neutrophils, and the Th1 cytotoxic response. There is little evidence that virulence factors specifically inhibit NK recognition or the cytotoxic effects of perforin/granulysin. This suggests that NK cells can exert their full anti-microbial response against *C. neoformans*.

Capsule:

Cryptococcus are encapsulated by a polysaccharide shell composed primarily of a 1,3-D-mannopyranose backbone with ß-D-xylopyranosyl and ß-D-glucuronopyranosyl attached to 6’ carbon - called glucuronoxylomannan (GXM) [35]. The capsule also contains galactoxylomannans (GalXM) formed with a galactose backbone and xyleomannan side chains, and mannoproteins [36,37].

This capsule is associated with virulence since mutants in genes responsible for capsule production usually have reduced virulence [38]. The capsule offers protection by inhibiting phagocytosis of *Cryptococcus*. Co-culture of the acapsular *C. neoformans* strain 602 with macrophage showed greater attachment and phagocytosis compared to the encapsulated strain 613 [39]. The addition of cryptococcal polysaccharide to media of the acapsular strain decreased macrophage attachment and phagocytosis [39]. The capsule provides *Cryptococcus* with a strong negative charge that could impair macrophage phagocytosis [40]. GalXM also induces apoptosis in macrophages through a Fas ligand mechanism and apoptosis in T cells through caspase 8 [41,42].

Encapsulated *Cryptococcus* also had reduced monocyte/macrophage release of pro-inflammatory cytokines, such as TNF-α [43]. TNF-α is required for the protective Th1 response against *Cryptococcus* [44]. Higher levels of TNF-α in cryptococcal infected mice were associated with greater recruitment of leukocytes to the lungs and reduced cryptococcal lung colony forming units (CFU) [45]. Anti-TNF-α therapy prevents CD4+ T cell recruitment to the lungs, and promotes cryptococcal dissemination to the brain [45]. Treatment of monocytes with cryptococcal polysaccharide also increased IL-10 production, which downregulated TNF-α and skew towards a Th2 response [46].

The capsule also acts as a sponge for complement. Complement allows for neutrophil swarming and macrophage cytotoxicity against *Cryptococcus*, and mice deficient in complement were more prone to succumbing to cryptococcal infection [47,48]. Patients with disseminated cryptococcosis were found to have reduced serum complement [49]. Low serum complement could provide an environment promoting further cryptococcal dissemination and hindering clearance. Injection of cryptococcal capsular polysaccharides reduced serum complement, suggesting that *Cryptococcus* could shed cryptococcal capsule that absorbs complement preventing immune clearance [49].

Capsule polysaccharide also directly impairs leukocyte migration by inducing neutrophil shedding of L-selectin. Incubation of neutrophils in the presence of GXM decreased surface L-selectin while increasing L-selectin in the supernatant [50]. GXM and GalXM

also bind to CD18 (β chain of lymphocyte function-associated antigen 1(LFA-1)) on neutrophils, which could impair migration [51].

Melanin:

Melanin is a brown/black pigment produced by certain isolates of *Cryptococcus [52,53]*. It is produced by the phenoloxidase activity of the organism, and is visible when *Cryptococcus* is grown on agar containing diphenolic compounds - such as birdseed or carrot extract [52]. In humans, *Cryptococcus* is thought to convert neurotransmitters - such as dopamine, norepinephrine, or L-3, 4-dihydroxyphenylalanine (L-DOPA) - indirectly into melanin [52].

Cryptococcal mutant that produce melanin are more pathogenic in mice compared to non-melanin producing mutants [54]. Melanin protects *Cryptococcus* against oxidative stress. *Cryptococcus* that produce melanin were more resistant to hypochlorite and were able to grow faster in broth containing permanganate [55]. *Cryptococcus* grown in the presence of L-DOPA also survived better in media containing nitric oxide or hydrogen peroxide compared to controls [56].

Laccase is the phenoloxidase that is responsible for melanin production. Cryptococcal mutants lacking laccase were less virulent in mice and caused lower fungal burden in the brain [57]. Cryptococcal isolates, from humans, that possessed higher laccase activity were able to survive longer in human cerebrospinal fluid [58]. The presence of laccase also promotes non-protective M2 macrophage polarization [59].

Mannitol:

Mannitol is a hexitol secreted by *C. neoformans*. The levels of mannitol correlated with CFU in infected rabbit's cerebrospinal fluid (CSF) [60]. A mutant strain of *C. neoformans* strain H99, that produced reduced levels of mannitol were more vulnerable to heat and osmotic stress [56,61]. The mutant strain was also less virulent in mice than wild type H99 [61]. Secretion of mannitol also prevented neutrophil killing [62].

Superoxide dismutase:

Cryptococcal mutants with disrupted superoxide dismutase were less virulent in mice than wild type or reconstituted strains [63]. They were also more susceptible to macrophage killing [63].

Phospholipase:

Cryptococcus also secrete phospholipases. Disruption of the phospholipase B gene increased murine survival, and reduced fungal burden [64]. The current hypothesis is that phospholipase B can disrupt host membranes, and allows *Cryptococcus* to penetrate into deep lung and brain tissue [65].

Metalloproteases:

Cryptococcal mutants lacking the secrete metalloproteases, Mpr1, were unable to cross an *in vitro* model of the blood brain barrier [66]. Mice infected with this mutant strain had prolonged survival and reduced cryptococcal burden in the brain [66].

Reproduction

C. neoformans can undergo both sexual and asexual reproduction. Pioneering work by Kwon-Chung found that cryptococcal sexual reproduction involves 2 mating type (a and α) and that their fruiting bodies lacked teliospore, making them more similar to *Filobasidium* [67]. Therefore the sexual form of *C. neoformans* is referred to as *Filobasidella [16]*. Growing mating type locus (MAT)a and MATα *Cryptococcus* together at 25°C causes formation of dikaryotic filamentous structures that develops into the basidium. In the basidium the nuclei fuse and undergo meiosis and produce haploid spores [68]. Alternatively, growing *Cryptococcus* with different MAT loci can result in a small subset of diploid monokaryotic yeast cells that are stable at 37°C [69]. A subsequent drop in temperature can cause these diploid cells to undergo filamentation where each segment is monokarytoic and diploid [69]. These filaments develop into a basidium that produces haploid spores containing either MATa or MATα. Asexual reproduction involves mitosis and the budding parent cells form daughter cells that share the same mating type. This process normally does not involve basidium formation. However, MAT α *Cryptococcus* in nutrient starved conditions can undergo haploid fruiting [70]. Haploid fruiting involves development of unclamped monokaryotic haploid filaments that develop into basidium and produces only haploid MAT α spores [70].

Species

Cryptococcus neoformans can be separated into subgroups based on serotyping and genetic variation. Serotyping is based on capsular antigen recognition by rabbit antisera [71]. Antisera A reacts with *Cryptococcus neoformans* var. *grubii* [72], while antisera D

reacts with *C. neoformans* var. *neoformans* [72]. Antisera B and C react with *Cryptococcus gattii.* There are clinical differences between var. *neoformans* and var. *grubii*. Var. *grubii* tend to causes more skin manifestations and infects older individuals[73,74]. Serotype D *Cryptococcus* are also more susceptible to heat killing, and found in temperate regions of the world compared to serotype A, which is found worldwide [75]. Overall *C. neoformans* var. *grubii* accounts for more than 79% of *C. neoformans* infections worldwide, which maybe due to combination of increased reservoir and higher virulence of var. *grubii* compared to var. *neoformans* [74]. There is also an uncommon hybrid serotype AD. While serotype A and D *Cryptococcus* are haploid in their yeast form, measuring the DNA content in serotype AD cells revealed that they are aneuploid or diploid [76]. As expected serotype AD cells were diploid in genes responsible for serotype antigen (*CLA4*, *CNA1*, and *GPA1*) [76]. Serotype AD strain were not as virulent in mice as serotype A - *C. neoformans* strain H99 - but they were still able to cause disease and mortality [76].

There is a correlation between the serotype and genotype of *Cryptococcus neoformans*. In general, VNI and VNII correspond to *C. neoformans* var. *grubii* (serotype A), while VNIII is serotype AD [77]. VNIV is *C. neoformans* var. *neoformans* (serotype D) [77]. Although there is a correlation between genotype and serotype, not all cryptococcal strain follow this pattern. For example, WM 628 is serotype D but genotype VNIII while WM 629 is serotype AD but genotype VNIV [78].

In my experiments, I test both serotypes of *C. neoformans.* Previous studies on NK signaling involved strain B3501, so I continued to use that strain to represent serotype D. In some experiments I also tested the clinically isolated strain 145, which represents serotype A [79].

Epidemiology

A higher proportion of *C. neoformans* patients are HIV positive or immunocompromised patients compared to *C. gattii* infections, where the majority of patients are immunocompetent [80]. *C. neoformans* is also more ubiquitous and found throughout the world, while *C. gattii* is more localized to sub-tropical and tropical regions [80]. Almost all clinical isolates (over 90%) of *Cryptococcus* from Canada and United States (excluding California) were serotyped as *C. neoformans var neoformans* [81]. Southern California has a much higher proportion of *C. gattii* at 41% compared to the 6% in the rest of the United States [81]. This unusually high levels of *C. gattii* could be associated with 3 novel tree species (Canary Island pine, American sweetgum, and Pohutukawa tree) present in California that were only recently identified to host *C. gattii* [82]. Tropical south American countries - such as Brazil - had much higher proportion of *C. gattii* isolates (80%), while colder countries - such as Argentina - only 10% of isolates were *C. gattii [81]*. European countries share a similar profile to Canada and United States, where only 5 out of 152 isolates from 9 countries were *C. gattii [81]*. Despite the proximity of Australia and New Zealand, 47.1% of isolates were *C. neoformans* in Australia compared to 92.3% in New Zealand [81]. In Asia, 94% of isolates from Japan were *C. neoformans* compared to southern Asian countries (Vietnam, Thailand,

Cambodia, and Nepal) where 50% of isolates were *C. gattii* [81]. In China, the majority of isolates (89-99%) were *C. neoformans*, with *C. gattii* isolates favoring southeastern provinces [83]. All clinical isolates - total of 6 - from central Africa were *C. gattii*, however the low number of isolates makes forming conclusions difficult [81].

The incidence of *Cryptococcus* in a country is highly dependent on the availability of highly active anti-retroviral therapy (HAART). In Australia and New Zealand 355 cases of *Cryptococcus* were identified between 1994 and 1997, resulting in a mean incidence of 6 per million people per year in Australia and 2.2 per million people per year in New Zealand [84]. Patients with AIDS increase the risk of cryptococcal infection. The incidence of *Cryptococcus* was 39.1 per thousand AIDS patients in 1994 and decreased to 13.5 per thousand AIDS patients in 1996 [84]. The reduction in cryptococcal infection in AIDS patients, between 1994 and 1996 could be attributed to the introduction of HAART in Australia in late 1991 [84].

The impact of HAART on cryptococcal infections was also observed in the United States. A study in Houston and Atlanta found the incidence of *Cryptococcus* dropped from 25-30 per thousand AIDS patients in 1993 to 2-6 per thousand AIDS patients in 2000 [85]. Between 1993 and 2000 the United States also saw drastic improvement in the management of AIDS patients due to HAART [86]. Similar drop in cryptococcal incidence were observed in Europe [87,88].

However in regions where HIV is endemic and access to HAART is limited, the incidence of *Cryptococcus* has persisted. In Uganda, a study between 1995 and 1999 showed the incidence of *Cryptococcus* at 40 per 1000 HIV infected people per year [89]. In South Africa, the rate was 14 per 1000 AIDS patients per year [90]. The global HIV prevalence in 2006 was an estimated 33,200,000 cases and 22,500,000 were in sub-Saharan Africa [91]. This suggests that cryptococcal disease is rampant in Africa. There are an estimated 600,000 deaths associated with cryptococcal meningitis in AIDS patients in sub-Saharan Africa annually [92].

Although the incidence of *Cryptococcus* has declined in region with access to HAART, the case-fatality rate of patients with *Cryptococcus* remains high. In patients with cryptococcal meningitis, treatment with fluconazole or amphotericin B alone results in a 10-week mortality rate of 14% and 18% respectively [11]. A combination therapy involving initial treatment with amphotericin B followed by fluconazole managed to reduce the 10-week mortality rate to 9.4% [11]. The probability of death at 4 years is 41% in HIV patients diagnosed with cryptococcal infection [93]. This case fatality rate is still higher than many diseases that are commonly recognized as public health hazards - such as mumps (1.4%), measles (1-10%), or malaria (1.3%) [94–96]. In areas lacking access to advanced therapies, the mortality rate approaches 100 percent, with a median survival of 27 days [97].

Overall, these data demonstrate that *Cryptococcus* is a global health concern, and studies into better treatment options are needed.

Manifestation of Disease:

Cryptococcal infections are acquired from the environment, specifically from the soil, tree bark, tree hallows, and bird droppings [98,99]. During sexual reproduction, *Cryptococcus* forms hyphae structures, that release spores. These spores are small enough to be inhaled and penetrate into deep lung structures. Desiccated yeast are also small enough to enter deep lung tissue [98]. The majority of infections in immunocompetent individuals causes mild or asymptomatic disease, with *Cryptococcus* being cleared or entering a dormant state [100].

The most common clinical manifestation of cryptococcal infection is cryptococcal meningoencephalitis (found in 77.2% of patients with cryptococcosis) [101]. The most common signs of cryptococcal meningoencephalitis are headache, neck stiffness, fever, papilledema, increased intracranial pressure, cranial nerve palsy, and decreased consciousness [102,103]. Cerebral cryptococcomas can press on surrounding tissues and cause seizures, hydrocephalus, and focal neurological findings [103]. The absence of fever or headache does not exclude cryptococcal encephalitis since patients can present with only dementia and myoclonus [104]. Computed tompography (CT) imaging of the brain only returned positive findings in 57% of patients [105]. The most common image findings are generalized cerebral atrophy (34% of imaged cases) possibly caused by HIV infection [105]. Hydrocephalus can also be found in 9% of cases, likely due to meningeal inflammation resulting in obstruction of CSF drainage [105]. Mass lesions were found in 11-25% of cases, but none of the findings were specific to

Cryptococcus and they could be mistaken for toxoplasmosis, infarction, leukoencephalitis, tuberculosis, abscesses, or cytomegalovirus [105].

Cryptococcus neoformans pneumonia is found in 8.2% of patients with cryptococcosis [106]. Presentation of pulmonary cryptococcosis is dependent on the level of immunosuppression. In HIV infected individuals with low CD4 counts (<100 cells/ul), pulmonary cryptococcosis usually accompanied disseminated disease, while higher CD4 counts are associated with localized lung disease [107]. In acquired immunodeficiency syndrome (AIDS) patients, pulmonary cryptococcosis symptoms included fever (94%), cough (74.1%), chest pain (42.9%), dyspnea (42.9%) and purulent sputum (14.3%) [106,107]. Hemoptysis was a rare findings (0-1%) [106,107]. Radiological findings included interstitial/alveolar infiltrate (42.9%), cavitation (14.3%), or pleural effusion (28.6%) [106].

Immunocompromised patients, with pulmonary cryptococcosis but without HIV infection, were less prone to disseminated disease than HIV patients (25% compared to 100% in AIDS patients) [106]. They also had a lower likelihood of cough, and dyspnea, but a higher likelihood of hemoptysis [106]. Radiologic findings of masses/nodules were more common, while interstitial infiltrates and cavitations were less common [106].

In a study of 42 HIV negative patients with pulmonary cryptococcosis, none of the immunocompetent patients developed disseminated disease [108]. A small subset of immunocompetent individuals could also spontaneously clear the pulmonary

cryptococcosis without medical treatment [109]. The clinical signs resembled the non-HIV immunocompromised patients with increased hemoptysis and decreased cough, and dyspnea [106,110]. Radiologic findings were also similar to the non-HIV immunocompromised group with fewer cavitations and more nodules [106].

There are also differences in clinical presentation between *C. neoformans* and *C. gattii*. *C. neoformans* focus on the meninges and rarely involves the cerebrum, while *C. gattii* infects both meninges and cerebrum [111]. *C. neoformans* is less common in the lungs, and more common in the blood, while *C. gattii* is found commonly in the lungs and rarely in the blood [111].

Less common clinical presentations include skin lesions[112], endophthalmitis [113], osteomyelitis and arthritis [114], cardiac valve and muscle involvement [115–117], pyelonephritis [118], and prostatitis [119–121].

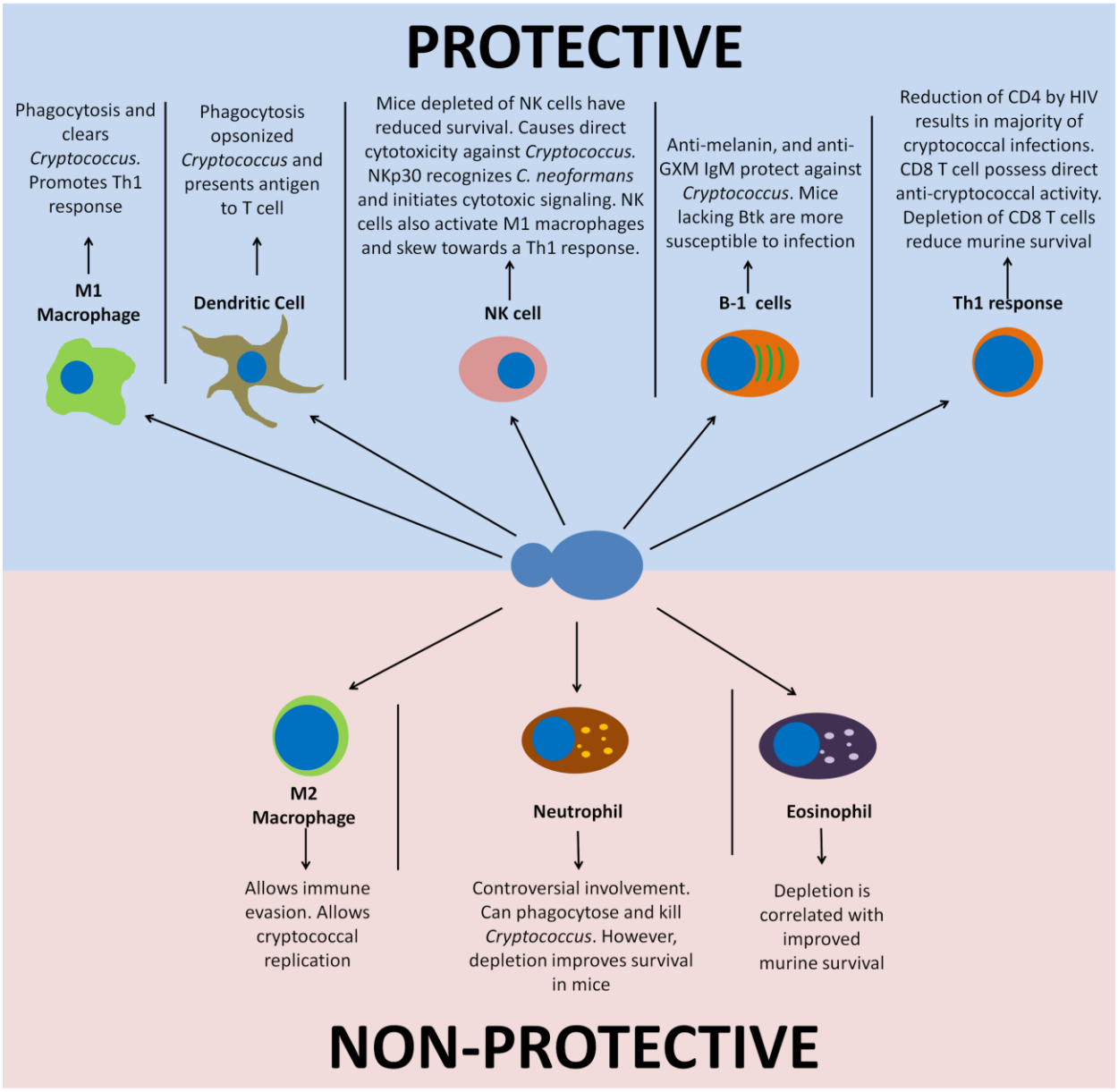


Figure 1-1: Immune cell involvement in cryptococcal clearance. Top: Leukocytes that provide protection against *Cryptococcus*. NK cells are the only innate immune cell that does not require opsonization or cytokine activation in order to directly killing *Cryptococcus*. Bottom: Leukocytes that do not protect against cryptococcal infection, or whose role is controversial.

Innate immune response to cryptococcal infection

Before the cryptococcal cell can invade the terminal respiratory tract and cause infection, it must navigate and survive the upper respiratory tract. The upper respiratory tract, involving the nasal cavity, pharynx, and larynx, acts as a filter and stops particles larger than 10 microns [122]. Particles larger than between 2-10 microns, the size the yeast form of *Cryptococcus*, are trapped in the bifurcating bronchial tree and are expelled by mucocilliary transport [122,123]. Particles smaller than 2 microns, such as desiccated *Cryptococcus* or cryptococcal spores, are deposited in the terminal respiratory tract and need to be cleared by the immune system [122].

Pathogens that enter the terminal respiratory tract are coated with complement [124,125]. In the case of *Cryptococcus* complement component 3 (C3) coats the capsule and assists phagocytosis via complement receptor 3 (CR3) [126]. Complement component 5 is also necessary, since mice deficient in complement component 5 had increased susceptibility to *Cryptococcus* [127]. In humans, a lack of complement proteins correlated with cryptococcosis [128].

Inside the body, fungal cells release chemotactic factor and induce the secretion of chemokines, resulting in recruitment of immune cells. The capsule of *C. neoformans* is chemotactic for neutrophils, as evidenced by the increase in neutrophils on the reverse

side of a Boyden chamber in response to encapsulated *Cryptococcus* [129]. GXM also upregulates release of chemotactic factors, such as: monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES from PBMC [130,131]. Intra tracheal injection of *C. neoformans* into mice also increased MCP-1, MIP-1α, and CXCL5 production [132,133].

Various immune cells target *Cryptococcus* (Figure 1-1), however their roles are dependent on the animal model being studied. In mice, alveolar macrophage (AM) were non-protective and their depletion resulted in reduced lung fungal burden, while AM depletion in rats resulted in increased fungal burden and dissemination [134,135]. This discrepancy could be the result of murine AM phagocytosing *C. neoformans* and acting as a protective niche and allowing intracellular fungal growth. In murine AM, cryptococci were found to replicate intracellularly both in the phagosomes and the cytoplasm, to the point of lysis of the AM [134,135]. Additionally, *in vitro* experiments, in mice, have shown that *Cryptococcus* can travel from one macrophage into another without exiting the cell [136]. This may allow the fungi to use the macrophage to avoid the extracellular immune system. Rat AM also phagocytose *Cryptococcus*, but had reduced intracellular replication [134]. Rat AM also exhibit greater anti-cryptococcal activity compared to murine AM [134].

Human AM also phagocytose *Cryptococcus* and cryptococcal replication can occur inside these AM [135]. *Cryptococcus* can also leave a macrophage by a lytic and non-lytic pathway. The lytic pathway may involve an excessive number of intracellular

Cryptococcus leading to rupture of the macrophage. The non-lytic expulsion of *Cryptococcus* is actin independent, but the mechanism, physiologic purpose, and if this benefits the fungi or the host is unknown [137,138]. Phagocytosis of *Cryptococcus* by monocytes in circulation also assists in cryptococcal trafficking and penetration of the blood brain barrier in a model called “Trojan Horse” [139].

Despite *Cryptococcus* exploiting the macrophage as a niche for replication, human macrophages also assist in cryptococcal clearance. Macrophages that phagocytosed opsonized *Cryptococcus* were able to produce IL-1 and present antigen to T cells [140]. These contrasting roles of macrophages can be explained by the presence of M1 and M2 macrophages. M1 macrophages respond to IFNγ and other Th1 cytokine, which activates the macrophage and allows it to kill *Cryptococcus*, while the M2 macrophages skew the immune system towards a non-protective Th2 response [141]. Cryptococcal infection alters the polarization of macrophages, first skewing towards M2, and then prolonged infection polarized towards M1 [142]. *Cryptococcus* promotes a M2 response by secretion of the virulence factor, Ssa1 [143].

M1 macrophage killing of *Cryptococcus* depends on IFNγ induced nitric oxide (NO) production [144]. Knockout of inducible nitric oxide synthase (iNOS) in macrophages increased the cryptococcal burden in mice [144]. Knockout of signal transducer and activator of transcription 1 (STAT1) prevented macrophage production of NO against an IFNγ secreting strain of *Cryptococcus [144]*. Since M2 macrophages do not mount a

robust NO response compared to M1 macrophages, M2 macrophages possess less anti-cryptococcal activity [142].

Neutrophils are the most abundant innate immune cell that is recruited to sites of infection [145]. Neutrophils have the capacity to kill *C. neoformans*, however their role in host defense is controversial. Various labs have shown using *in vitro* studies that neutrophils phagocytose and kill *Cryptococcus* [146,147]. *In vitro* data also shows neutrophils release neutrophil extracellular traps (NETs) in response to *C. neoformans* [148]. However, other groups have shown that *in vivo* mice with depleted neutrophils have increased survival to cryptococcal infection [149]. In neutrophil depleted mice, there was an increase in leukocyte recruitment and also an increase in IL-10 [149]. Therefore, the improved survival could be the result of reduced inflammation or improved leukocyte recruitment. While IL-10 is traditionally thought of as a Th2 promoting cytokine, IL-10 - when in conjunction with IL-18 - can enhance IFNγ secretion by NK cells [150]. *In vitro* studies also showed that IL-10 enhances the expression of genes involved in NK cytotoxicity and migration [151]. Additional studies are required to determine if enhancing neutrophil function will improve anti-cryptococcal response.

The presence of eosinophils is also detrimental to murine survival [152]. In mice, cryptococcal infection induced eosinophilic pneumonia [153]. There is also a case report of a patient with eosinophilia and disseminated cryptococcosis. It is likely that the eosinophilia and cryptococcal infection were related since the eosinophilia cleared with

treatment of the cryptococcal meningitis [154]. However, is was unknown if the eosinophilia was detrimental or beneficial to the patient.

NK cells play a vital role in host defence against *Cryptococcus*, and they will be discussed in their own section. While there are other innate immune cells, little is known about their anti-cryptococcal ability.

The adaptive immune response is crucial to defend against *Cryptococcus* and the Th1 response is likely the most important factor in the ability of patients to clear *C. neoformans*.

T cell are essential for cryptococcal clearance [155,156] and patients lacking CD4 T cells, especially due to HIV, are most susceptible to cryptococcal infection; suggesting that T cells are the most important immune cell in cryptococcal clearance [157,158]. This is likely due to the protective Th1 cytokines produced by CD4 T cells [159,160]. Adoptive transfer of CD4 T cells from *Cryptococcus* immunized mice conferred protection in the recipient [161]. CD4 T cell directly kill *Cryptococcus* through a granulysin dependent mechanism [162]. Similarly, CD8 T cells also directly killing *Cryptococcus* through a granulysin dependent mechanism [163]. Depletion of CD8 T cells in mice decreased their survival to cryptococcal infection [164]. CD8 T cells also secrete IFNγ, which activates macrophage mediated killing of *Cryptococcus [165]*.

The Th1 anti-cryptococcal response needs to be initiated by dendritic cells (DC). The recruitment of monocytes to the lung and their differentiation into DC is essential to

survival of mice infected with *C. neoformans [166].* DC are primarily focused on activating the adaptive immune response, rather than rapid innate immunity.

The Th1 response is essential for cryptococcal clearance. Dendritic cells stimulated with *Cryptococcus* produced Th1 cytokines - IL-12 and IL-23 [167,168]. Dendritic cell recognition and uptake of *Cryptococcus* is dependent on mannose receptor (MR) and Fcγ receptor II [169]. Mannoproteins from the capsule of *Cryptococcus* causes DC maturation through MR [170]. The addition of blocking antibodies against mannose receptor blocks this maturation and production of TNFα and IL-12 [170]. Both IL-12 and TNFα contribute to cryptococcal clearance. Addition of IL-12 increases TNFα production and TNFα enhances Th1 skewing of T cells [171,172]. The ability of DC to bind, and phagocytose *C. neoformans* required opsonization of *Cryptococcus* by complement or antibodies [173,174]. However the role of opsonins varies with cryptococcal species, as binding to *C. gattii* was opsonin independent [175].

The role of B cells and antibodies have been shown to enhance immunity against *Cryptococcus*. B cells make up the largest group (15-30%) of the leukocytes in the lungs of mice infected with *Cryptococcus.* B-1a B cells secrete IgM antibodies that bind and clear *Cryptococcus [176]*. Mice with defective Bruton's tyrosine kinase (Btk) have increased susceptibility to cryptococcal infection [177]. Severe combined immunodeficiency (SCID) mice reconstituted with only T lymphocytes had increased fungal burdens, compared to SCID mice reconstituted with both T and B cells [178]. Serum from healthy humans possess natural IgM, IgG, and IgA against GXM expressed

on *Cryptococcus [179]*. Mice produce a large amount of IgM and IgG against cryptococcal melanin [180], and passive immunization with anti-melanin antibodies reduced cryptococcal fungal burden in murine cryptococcal infections [181]. Serum IgM protected mice against cryptococcal meningitis [182]. Adult humans possess natural antibodies that target GXM [183,184]. Transplant patients with lower levels of anti-GXM IgM were more susceptible to cryptococcal infection [185].

In a healthy human, Th1 CD4 T cells are likely the most important cell against cryptococcal infection. However, AIDS patients - who are at great risk of cryptococcal infection - have suppressed adaptive immune responses. Since, my research aims to enhance immune therapy, my ideal cell is a rapidly activated innate leukocyte that possesses receptors that directly recognizes *Cryptococcus* and induces cytotoxicity. These criteria are met by NK cells. This thesis will focus on the role of NK cells in protecting against cryptococcal infection.

NK cells:

Overview

In 1974, while investigating IgM and complement lysis of sheep red blood cells (sRBC), Dr. Thornthwaite observed an unusual cell that could lyse sRBC in an abnormal manner [186]. Complement lysis caused RBC to shrivel and remain on the microscopy slide [186]. However, an unidentified cell was found to release compounds that were completely obliterating the surrounding sRBC. Additionally, these cells could lyse sRBC in the absence of complement. They were termed complement independent plaque

forming cells [186] and were later renamed in 1975 to natural killer cells [187]. The term “natural” was given due to their ability to kill targets without prior exposure [187].

NK cells develop from the common lymphoid progenitor in the bone marrow [188]. There is a lack of consensus on when to demarcate a new NK precursor, however the expression of IL-15R is an important step. IL-15R is required for survival and maturation of NK precursors [189]. Expression of IL-15R is part of the first stage towards an NK lineage. As this precursor develops, it migrates to the T cell area in secondary lymphoid organs and gains the expression of IL-2Rβ, and are considered stage 2 [190,191]. *In vitro*, stage 2 cells can still differentiate into DC and T cells [191]. However, upon expressing LFA-1 and progressing the stage 3 these precursors lose the ability to differentiate into DC and T cells [190]. These progenitors then develop into maturing $CD56^{bright}$ cells that give rise to a mature $CD56^{dim}$ population [192]. $CD56^{dim}$ cells circulate in the blood and survey for tumor, virally infected cells, and microbes.

NK cells are best known for their anti-tumor capabilities. NK tumor killing is a vast and extensively explored field of NK biology. In brief, NK recognition of tumor targets depends on both activating receptors and inhibitory receptors. Inhibitory receptors bear immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic domains. Engagement of these ITIM receptors results in reduced NK cell activation and adhesion to their target. This is achieved by both dephosphorylation and phosphorylation of different proteins important in NK cell signaling. ITIM signaling recruits phosphatases - such as src homology 2 domain phosphatase (SHP)1 and SHP2 - through a β-arrestin 2

dependent mechanism [193]. SHP1/2 then dephosphorylates proteins necessary for NK activation, such as Vav1 [194]. In contrast, engagement of inhibitory CD94-NKG2A results in phosphorylation of Crk, which dissociates it from c-Cbl [195]. In activation pathways c-Cbl-Crk complexes are important in actin remodeling [195]. The inhibitory receptors also allow viruses to evade NK cytotoxicity. Evasion of NK cell cytotoxicity involved encoding homologs of major histocompatibility complex (MHC) class I or MHC-independent molecules, or upregulating the expression of MHC class I molecules in the host cell [196–198]. While inhibitory NK receptors are important in self-tolerance and viral evasion, it is unclear what role they play in anti-microbial killing.

Activating NK receptor can associate with adaptors that contain activation motifs. Of particular relevance to microbial killing are a subset of natural cytotoxicity receptors (NCRs). NCRs are a family of NK receptors that belong under the Ig-superfamily [199]. The subset of NCRs in humans that have been found to target microbes are NKp30, NKp44, and NKp46. In tumor killing NKp30 has been found to bind to B7-H6, which is expressed on the surface of tumor cells and not healthy adult tissue [200]. NKp30 also binds human leukocyte antigen (HLA)-B-associated transcript 3, which is a nuclear factor released by tumor cells. NKp30 and NKp46 bind to heparan sulfate proteoglycans, which is non-specific for tumors. This subset of NCRs signals through their association with immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor proteins. NKp46 associates with FcRγ, and CD3ζ [201], NKp30 associates with CD3ζ [202], and NKp44 associates with CD3ζ and DNAX activation protein of 12kDa (DAP12) [203]. Phosphorylation of the ITAMs in the adaptor proteins allows recruitment of downstream

effector protein - such as Syk/Zap70 - that proceed to activate the NK cells and initiate cytotoxicity.

NK cell mediate microbial killing, excluding fungi

NK cell microbial killing can occur via direct and cytokine-mediated pathways. The direct pathway only requires the activity of the NK cell, while the cytokine-mediated pathway requires NK cells activating other leukocytes that then target the microbe.

NK cells recognize a variety of microbes including bacteria, protozoa, and fungi and cause both direct and cytokine mediated cytotoxicity. Microbial recognition involves a combination of NCR and canonical pathogen associated molecular pattern receptor (PAMPR) - such as toll-like receptors (TLR). NK cells express TLR 1-11 [204], and bacterial antigens induced NK IFNγ and α defensin secretion, in TLR2 dependent pathway [205]. TLR 3, 5, 7, and 9 agonists also directly cause NK proliferation and IFNγ or TNFα secretion [206,207]. Bacterial PAMPs can also prime the NK cells for activation by stimulating other leukocytes to release activating cytokines [208]. For example, *Listeria monocytogenes* infections cause macrophages and DC to secrete TNFα, IL-1β, IL-12, and IL-18, which activates NK cells [209–211]. TLR induced activation of NK cells results in the release of IFNγ, and does not cause direct cytotoxicity. NCR recognition of bacteria does cause direct cytotoxicity. Human primary NK cells kill *Mycobacterium bovis* in a contact dependent manner [212]. Depletion of cytolytic granules or small interfering RNA (siRNA) knockdown of perforin and granulysin inhibited killing of *M. bovis*. siRNA knockdown of NKp30, NKp44, NKp46, or NKG2D all inhibited killing of *M.*

bovis [212]. Killing of *M. bovis* depends on the activation of all 3 mitogen-activated protein kinase (MAPK) cascades (extracellular signal-regulated kinases (Erk), Jnk, and p38) [212]. NKp44 also binds to bacterial cell wall components from *Nocardia farcinica, and Pseudomonas aeruginosa* [213].

Cytokine activation of NK cells also occurs in protozoan infections [214,215]. IL-12 administration to mice infected with *Plasmodium chabaudi* induces protection by activation of NK cells [216]. However the protective effects were due to IFNγ release, and not direct cytotoxicity [217]. Cytokine activation of NK cells also occurs with *Plasmodium falciparum* and *Leishmania major* [218,219]. NK cell also protect against *P. falciparum* by recognizing P. falciparum erythrocyte membrane protein-1 via NKp30 and lysing the infected RBC [220].

NK mediated fungal killing

Cytokine mediated fungal cytotoxicity

NK cells can act as a regulatory cell and coordinate the immune system to mount an anti-fungal response. Mice deficient in NK cells and T cells had reduced survival and were prone to esophageal and gastric candidiasis [221]. A component of NK cell's role in *Candida* is NK cell activation of macrophages, since mice depleted of NK cell by anti-asialo GM_1 suffered impaired phagocytosis of *Candida albicans* by splenic macrophages [222]. NK cell recruitment to the lungs is also essential to murine survival against *Aspergillus [223]*. In neutrophil deficient mice, IFNγ from NK cells was crucial to

surviving *Aspergillus* [224]. NK cytokine mediated killing highlights the importance of the Th1 cytokine IFNγ in protecting against fungal infection.

Direct fungal cytotoxicity

In vitro experiments have shown that NK cells play a crucial role in killing *Aspergillus fumigatus [225,226],* and *Paracoccidioides brasiliensis [227]*. *A. fumigatus* hyphae activated NK cells and increased surface expression of CD69 [226]. Both unstimulated and IL-2 pre-stimulated NK cells exhibited direct killing of *A. fumigatus* hyphae [226]. Killing of *A. fumigatus* hyphae was found to be perforin dependent [226]. NK cells also possess direct cytotoxicity against fungi in the yeast phase. Co-culturing IL-2 stimulated NK cells with germlings of *A. fumigatus*, also causes an anti-fungal response [225]. The initiation of this cytotoxic response is contact mediated [225]. However, unlike the response to hyphae, NK cells did not require perforin degranulation [225]. Instead the cytotoxicity was dependent on the direct cytotoxic activity of IFNγ [225]. NK cells are also cytotoxic against 6 different isolates of the yeast form of *P. brasiliensis* [227]. In humans, there is a correlation between reduced NK cells cytotoxic activity and fungal infections. A systemic lupus erythematous patient treated with corticosteroid had reduced circulating NK cells, impaired NK cell cytotoxicity, and also a *Trichophyton rubrum* infection [228]. Another patient with reduced NK cell activity also developed disseminated *Aspergillus* infection [229]. Direct NK fungal cytotoxicity demonstrates NK cells' capability to release anti-fungal effector molecules.

Anti-fungal receptors on NK cells, excluding *Cryptococcus*

NK cell recognition of fungi is less explored compared to tumor recognition. However, NK cells possess some of the same receptors that are used by other innate immune cells to recognize fungal pathogens. Other innate immune cells recognize fungal targets by C-type lectin receptors (CLR) [230], Nod-like receptors (NLR) [231] [232], scavenger receptors, integrin[233–235], and TLR[236,237] [238,239]. PAMP in fungi are expressed on the cell wall of the organism and include β-glucans, chitin, mannan, and mannosides [240].

β-2 integrins are expressed on NK cells, and in other leukocytes β-2 integrins have been shown to recognize β-glucan on fungi. β-glucans are polysaccharides of β-D-glucose bound to each other through β1,3 and β1,6 glycosidic linkages. β-glucans on *C. albicans* are also recognized by CD11c/CD18 integrins [241]. Expression of CD11c/CD18 on human embryonic kidney cells 293 (HEK293) cells increased their adherence to and phagocytosis of *C. albicans* [241]. Eosinophils also recognize β-glucans on *Alternaria alternata* by CD11b/CD18, and antibody blockage of CD11b/CD18 on eosinophils inhibited degranulation [242].

NK cells also express various TLRs [243]. TLR 2, and 4 recognize fungal zymosan, *O*-linked mannan, fungal DNA, and phospholipomannan. TLR2 is recruited to macrophage phagosomes containing zymosan coated particles [244]. TLR2, TLR4[245], and CD14 activated NFkappaB after *A. fumigatus* stimulation [246–248]. Mice deficient in TLR 4 had increased *C. albicans* growth, and human peripheral blood mononuclear cells (PBMCs) stimulated with *C. albicans* had reduced cytokine secretion when treated with

blocking anti-TLR 2 antibody [249,250]. MyD88 deficient mice were more susceptible to fungal infections [251]. However, in a human study of 48 patients with IRAK-4 deficiency and 12 patients with MyD88 deficiency no increased susceptibility to fungal infections was detected [252]. This suggests that there are redundant PAMPR that can compensate for TLRs.

NLR recognized intracellular fungal targets. Nod-like receptor protein (NLRP)3 is required for protection against *Candida albicans* and *Aspergillus fumigatus* [253,254]. NLRs augments signaling from TLR2. TLR2 signaling leads to production of pro-IL-1β, which is then cleaved into its active form by NLRP3 activation of caspase 1 [255]. The fungal ligand recognized by NLRP3 is unknown.

Importance of NK cells in cryptococcal immunity

As detailed previously, *Cryptococcus* possesses mechanisms to subvert macrophage cytotoxicity [135]. Although Th1 cytokine stimulated macrophages are effective, it is part of a slower adaptive responsive. On the other hand, NK cells exhibit direct, rapid, and opsonin-independent cytotoxicity against *C. neoformans*.

Injection of *C. neoformans* increased the recruitment of NK cells to the spleen and lungs [256]. NK cells also associate with cryptococcomas in the brain [257]. In order to test the role of NK cells, earlier studies involved adoptive transfer of nylon wool nonadherent cells, with or without anti-asialo GM1 antibody depletion. Using this technique, depletion of NK cells increased the fungal burden of *C. neoformans* in the lung, liver, and spleen of mice. This effect was seen as early as 4 days post-transfection [256]. Another study found depletion of NK cells with anti-asialo GM1 or anti NK1.1 elevated CFU in the

lungs of mice as early as 24 hours post-cryptococcal injection [258]. NK cell depletion also reduced the survival of the mice [258]. Purified NK cells from humans and mice were able to kill cryptococcal cells *in vitro* [259–261]**.** *Cryptococcus* activates NK cytotoxic signaling and knockdown of receptors or cytotoxic molecules in NK cells inhibited cryptococcal killing [262–264]. NK cell killing of *Cryptococcus* is granule dependent. Beige mice (mutation in *Lyst)* have impaired NK granule mediated killing, as well as impaired neutrophil and macrophage activity [265,266]. Beige mice were also more susceptible to cryptococcal infection [265]. It is likely that the increased susceptibility is due to lack of NK cell function since neutrophils and macrophages isolated from beige mice were able to phagocytose and kill *Cryptococcus* at a same or better efficiency compared to control [265]. Together these studies show that NK cells provide rapid anti-cryptococcal immunity.

Mechanisms of NK anti-cryptococcal activity

NK cells possess both cytokine mediated and direct anti-cryptococcal activity.

Cytokine mediated anti-cryptococcal cytotoxicity

IFNγ is a key cytokine in the clearance of *C. neoformans*. IFNγ is predominantly produced by NK cells, NKT cells, and Th1 CD4 T cells [267]. IFNγ skews the T cells towards a Th1 response. IFNγ also enhances anti-cryptococcal activity of macrophages by stimulating the production of TNFα and release of nitric oxide [268]. NK cells from SCID mice secreted IFNγ and activated macrophage killing of *C. neoformans* [269]. IFNγ production also reduced the CFU in lungs and brain, and improved murine survival by reducing the Th2 response [270]. Blocking IFNγ with inhibitory antibodies, increased

the CFU of *Cryptococcus* in lungs, liver, and spleen [271]. It should be noted that IFNγ production by NK cells required stimulation with IL-12 and IL-18. The production of IL-12 and IL-18 in HIV patients is limited [272,273] - therefore NK mediated IFNγ production in HIV patient may also be limited. This suggests that direct NK cell cytotoxicity against *Cryptococcus* may be more important in patients suffering from AIDS. NK cells also produce TNFα, but *C. neoformans* inhibits its secretion and activity [274].

Direct anti-cryptococcal cytotoxicity

In order to study direct anti-cryptococcal cytotoxicity with NK cells, a human *in vitro* system is preferred over a murine model, since direct anti-cryptococcal activity depends on human specific proteins (NKp30 and granulysin).

Direct anti-cryptococcal activity is due to NK cells killing *Cryptococcus*, rather than NK cells slowing or freezing cryptococcal growth because in a limiting dilution assay co-culture of NK cells and *Cryptococcus* reduced the number of *Cryptococcus* positive wells [257]. Additionally, co-culture with NK cells increased the proportion of propidium iodide positive *Cryptococcus* [257]. Also stains for metabolic activity found that *Cryptococcus* in conjugates with NK cells had increased death compared to free floating *Cryptococcus* [260].

Transwell experiments showed that NK cells only kill *Cryptococcus* when they shared the same compartment, suggesting contact mediated killing [275]. Contact mediated killing of *Cryptococcus* occurs through antibody dependent and independent pathways.

Evidence of antibody dependent cytotoxicity is seen when addition of anti-cryptococcal polysaccharide antibodies enhanced the cytotoxicity of murine NK cells [276]. However, in the absence of antibodies, NK cells still possessed direct cytotoxicity. Using an NK cell line - YT cells - that lack CD16 (FcγRIII) and media that contain heat inactivated serum, the NK cells were still cytotoxic against *C. neoformans* [277]. Studies found that certain NCRs - that are important in anti-tumor and anti-viral response - are also important in cryptococcal killing. NK cells from patient susceptible to *Cryptococcus* - i.e. HIV patient - had reduced levels of the NCR NKp30 [278]. NKp30 was also found to polarize towards the site of contact with *C. neoformans* [279]. Antibody inhibition or siRNA knockdown of NKp30 blocked NK cell cytotoxicity against *C. neoformans [280]*.

Mechanisms of direct NK anti-cryptococcal activity

Cytotoxic granules are required for direct NK cell-mediated cryptococcal killing, since granule depletion with $SrCl_2$ prevented NK cell killing [281]. siRNA knockdown of perforin inhibited NK cytotoxicity [282]. Blocking NKp30 also reduced perforin degranulation [283]. This suggests that NK cells kill *Cryptococcus* via the exocytosis of perforin granules. Although NKp30 associates with CD3ζ, the involvement of CD3ζ in anti-cryptococcal killing has not been verified. NKp30 was found to signal through phosphoinositide 3-kinase (PI3K) [283] (figure 1-2). Inhibition of PI3K using siRNA or small molecule inhibitors prevented NK killing of *Cryptococcus* [284]. Erk was also found to be activated by cryptococcal stimulation [284]. Inhibition of PI3K prevented Erk activation, suggesting that PI3K is upstream of Erk [284]. Both PI3K and Erk are required for perforin polarization and degranulation [284]. Src family kinase (SFK), specifically Fyn and Lyn, are required for cryptococcal killing [285]. The receptor

required to activate SFK is unknown, however NKp30 associates with CD3ζ which requires SFK for activation. siRNA knockdown and small molecule inhibitor studies found that SFK are required to activate the PI3K → Erk signaling cascade [285]. The signaling pathways involved in cryptococcal killing is diagramed in figure 1-2.

Perforin mediated cytotoxicity

Perforin was first observed by electron microscopy in 1980 to form pore-like lesions on the membrane of K562 target cells following antibody dependent cell-mediated cytotoxicity by NK cells [286]. Cytotoxic signaling causes perforin granules to cluster close to the microtubule-organizing center (MTOC), which then move towards the site of contact [287]. Once the MTOC is at the site of contact, the perforin-containing granules move along the microtubule towards the immune synapse [288]. The granules eventually fuse with the immune synapse and release the cytotoxic perforin. The fusion of granules with the cell membrane involves a complex of trafficking molecules such as MUNC13-4, MUNC18-2, syntaxin 11, and SNAP-23 [289]. On tumor targets, perforin inserts into the cell membrane, oligomerizes, and forms pores. There was a debate on whether the pores were sufficient to allow other cytotoxic molecules - such as granzymes into the cell. Recent experiments have swung the model in favor of perforin pores being a sufficient size to allow granzymes directly into the target cell, without the need for endocytosis [290]. It is unclear if granzymes are involved in NK cell-mediated cryptococcal killing and how perforin would bypass *Cryptococcus's* cell wall and introduce the granzymes. The introduction of granzymes into tumor targets induce

apoptosis through cleavage of caspases [291]. Additional research is need to determine if similar targets exist in *C. neoformans*.

Rationale for investigating signaling

Studying signaling pathways can impact clinical research by discovering targets for immunotherapy. The identification of PI3K and Erk has provided potential targets for immunotherapy. Erk activity can be enhanced by small molecule inhibitors, such as fisetin and resveratrol [292]. Likewise PI3K activity can also be enhanced by cell permeable peptides [293]. However, the problem with altering PI3K and Erk activity, is that these molecules are involved in a tremendously large number of signaling pathways - such as apoptosis, protein, translation, proliferation, glucose metabolism, cytoskeletal remodeling, and differentiation [294,295]. Enhancing PI3K or Erk is likely to impact cell function outside of cytotoxicity. Therefore additional research is required to determine the anti-cryptococcal signaling pathways that are limited to cytotoxicity, and activate or are activated by PI3K → Erk. Targeting pathways involved in only cytotoxicity will reduce the toxicity of immunotherapy.

While NKp30 has been identified as an anti-cryptococcal receptor [262], evidence exists that there might be other receptors that are also involved in cryptococcal killing. NK degranulation is dependent on multiple receptor signals [10]. The process of degranulation involves the polarization of granules to the immune synapse, and the fusion of the granules with the cell membrane [296]. If either of these steps is missing then cytotoxicity would be limited due to lack of degranulation or non-targeted exocytosis. The fusion of granules with the cell membrane in NK cells is dependent on

signaling through ITAM adaptor proteins - which NKp30 utilizes [297]. In antibody-dependent cell-mediated cytotoxicity (ADCC) the polarization of granules to the immune synapse is dependent on integrins, such as LFA-1 [298]. Although NKp30 may provide the signal for granule fusion in cryptococcal killing, it is unclear if an integrin is causing granule polarization. If there is an integrin involved it is unlikely to be LFA-1 since siRNA knockdown of CD18 did not inhibit NK-cryptococcal killing [299]. Studying anti-cryptococcal signaling can help to inform the adhesion molecule involved.

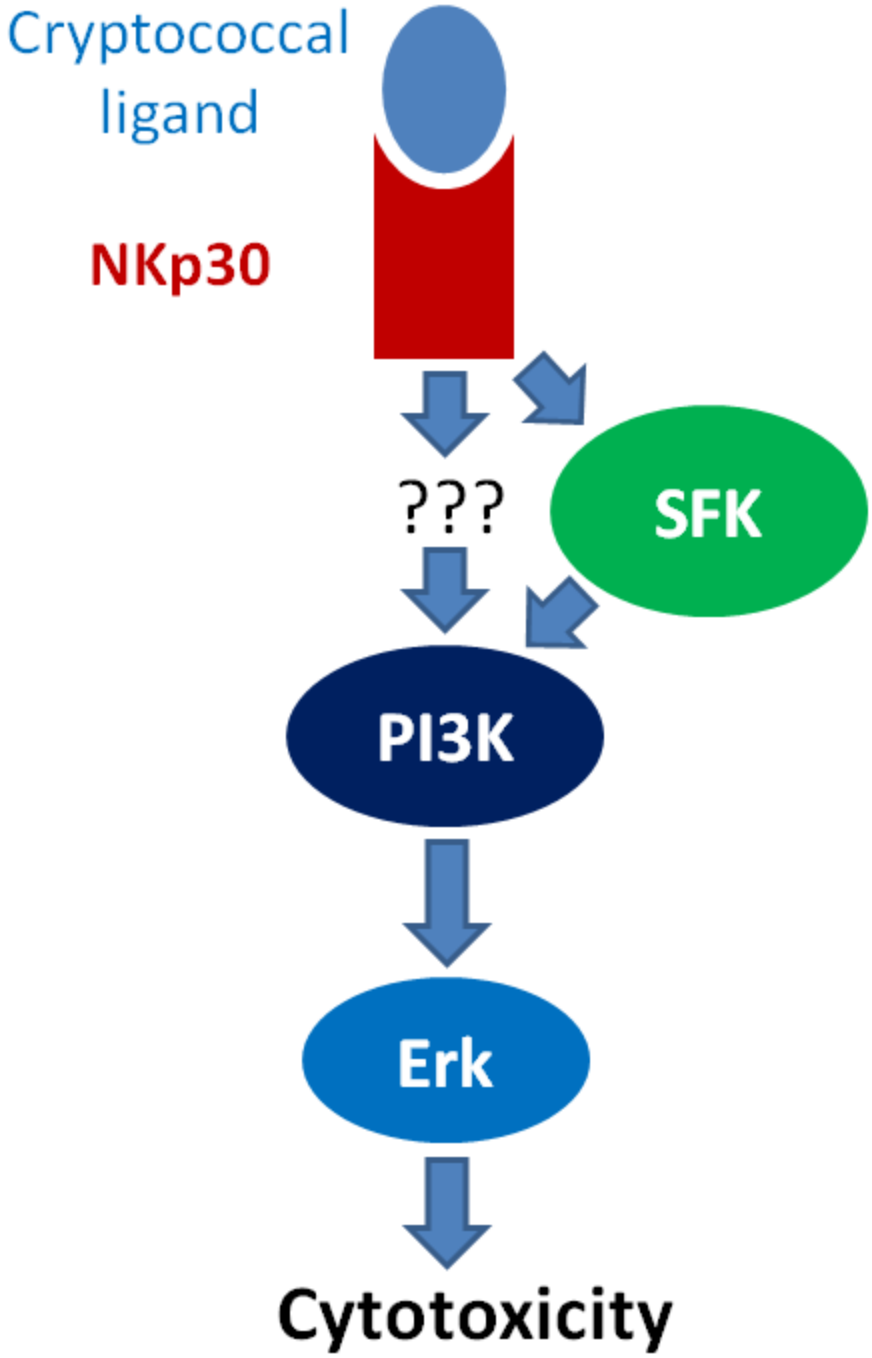


Figure 1-2: Signaling pathway in NK mediated cryptococcal killing. NKp30 recognizes *Cryptococcus* and activates a PI3K → Erk pathway. SFK are also required for the activation of PI3K → Erk. It is unclear what receptor activates SFK.

Integrin

Integrins are a class of cell surface proteins that play a major role in cell-cell, and cell-matrix adhesion. Integrins are also involved in signaling and cell migration. Each integrin is made up of an α and β subunit. A total of 18 α and 8 β subunits have been identified in vertebrates, and these subunits form 24 different α-β pairs. This thesis will explore the role of integrins in NK cell anti-cryptococcal killing.

The majority of immune function associated with integrins are due to the β-2 (CD18) class of integrins. β-2 integrins are restricted to leukocytes, and they pair with four exclusive α chains (CD11a (αL), CD11b (αM), CD11c (αX), and CD11d (αD)). β-2 integrins are involved in phagocytosis, migration, leukocyte development, conjugate formation, and direct cytotoxicity [300–303]. β-2 integrins are especially important in NK-tumor conjugate formation and cytotoxic signaling [303]. Blockage of LFA-1 (CD11a/CD18) on NK cells prevent cytotoxicity against K562 and Daudi target cells [304]. With IL-2 expanded NK cells, expression of intercellular adhesion molecule (ICAM)-1 on SC2 *Drosophila* cells was sufficient to induce perforin polarization to the IS and target lysis [303]. The ligand used to cross-link LFA-1 can also impact the function of LFA-1. In resting NK cells, ICAM-1 ligation of LFA-1 was not sufficient for perforin granule fusion with the plasma membrane [10,305]. However, ICAM-2 cross-linking of LFA-1 induced perforin degranulation [305]. The importance of β-2 integrins in NK cell-mediated tumor killing suggested that β-2 integrins may also be involved in NK cell fungal killing. This hypothesis was supported by *in vitro* data showing that integrins can

bind to soluble β-glucan from fungal targets [301]. However, when CD18 was blocked by antibodies or reduced by siRNA knockdown in NK cells, there was no impact on anti-cryptococcal killing [306].

Another class of integrins that mediates leukocyte cytotoxicity are β-1 integrins. β-1 integrins are the largest integrin class, and is necessary for migration, phagocytosis, and cytotoxic signaling in leukocytes [307–309]. Resting NK cells express α 3, 4, 5, and 6 integrin chains that form heterodimers with β-1 [310]. Upon IL-2 treatment, NK cells also upregulate α1 β1 and α2 β1 [311]. α4/β1 (very late antigen 4 or VLA 4) and α5/β1 (VLA 5) are the best studied β-1 integrins in NK cell migration and cytotoxicity. VLA 4 on NK cells bind to ICAM-1 and vascular adhesion molecule (VCAM)-1 on endothelial cells and facilitates NK chemotaxis [312]. Fibronectin also binds to VLA 4 and VLA 5 on NK cells and activates a Pyk2/Paxillin signaling pathway, which is involved in polarization of the MTOC and cytotoxicity [313,314]. Cross-linking VLA 4 and VLA 5 with fibronectin or antibodies increased NK cell intracellular calcium concentration and tumor cytotoxicity [315]. However, there is conflicting data from a different study where cross-linking VLA 4 and VLA 5 decreased CD16 mediated phospholipase D activation and reduced degranulation - measured by benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) esterase release [316]. It is unclear what causes this discrepancy.

Ligation of β-1 integrins on NK cells also cause cytokine secretion. β-1 integrins can activate a Ras→ Erk pathway that is important for IFNγ secretion [317]. They also control IL-8 secretion through a Vav→ Rac1→ p38 pathway [318]. In T cells, β-1

integrins were found to enhance degranulation against tumor targets by activating a SFK → Erk signaling pathway [319]. β-1 integrins also recognize β-glucans and triggers oxidative bursts in PBMCs [320]. The conflicting data on the involvement of β-1 integrins in NK cytotoxicity and the fact that the role of β-1 integrins in NK fungal killing is unexplored, makes further study of β-1 integrins imperative for understanding NK cell biology.

Integrins signaling leads to the assembly of a Src and focal adhesion kinase (FAK) complex that also consists of integrin linked kinase (ILK), and Crk-associated substrate (Cas) [321]. This signaling complex activates two major downstream pathways, MAPK and PI3K (Figure 1-3). Src phosphorylates Cas and allows Cas to associate with Crk, which increases Rac activity [322]. Rac then activates MAPK cascades through a PAK1-MEK1-MAPK pathway [323]. Src also phosphorylates FAK [321]. Phosphorylated FAK, along with Crk recruits PI3K and leads to Akt activation [324]. In this thesis, I will explore if *C. neoformans* stimulates β-1 integrins and if that stimulation leads to activation of the PI3K→ Erk cascade.

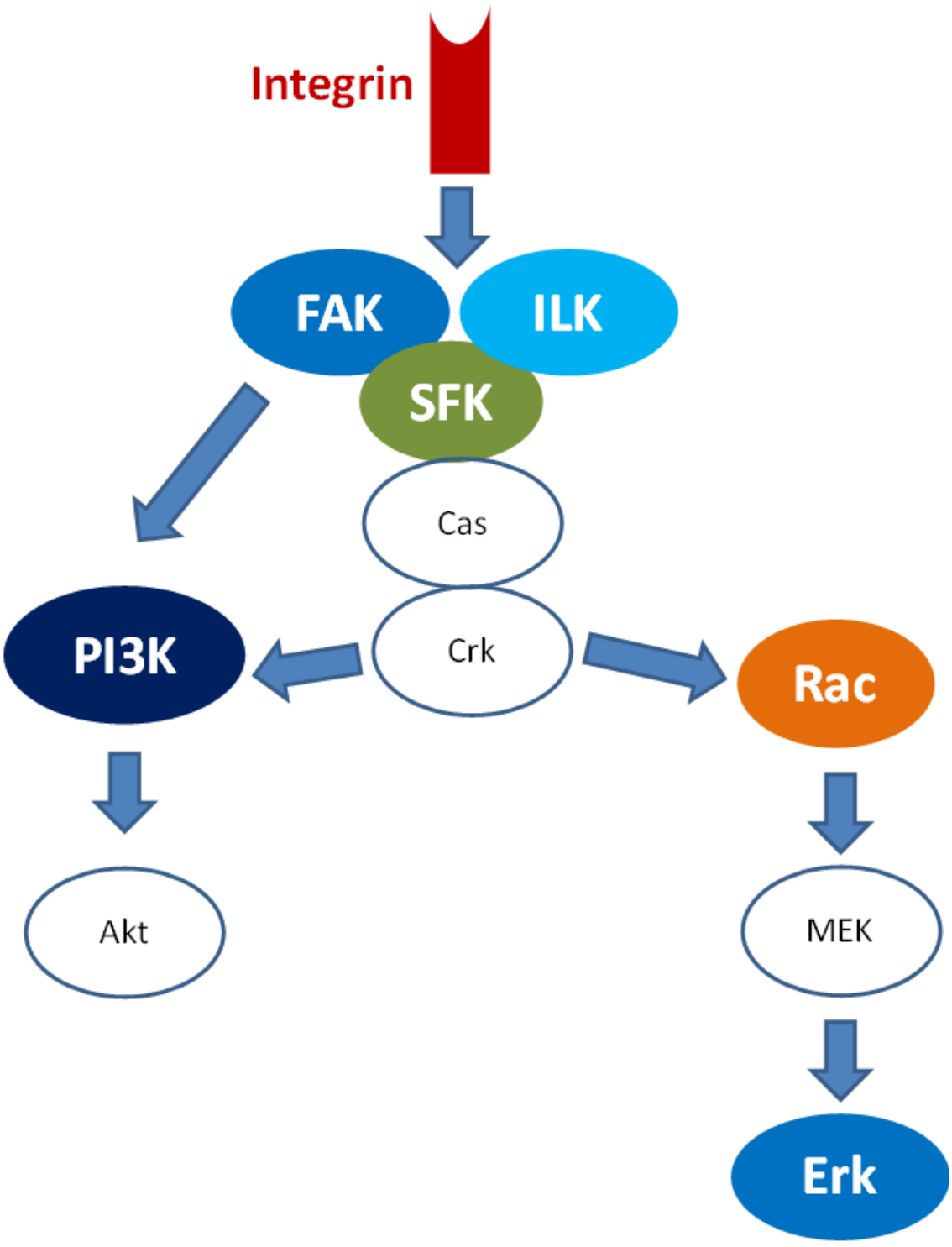


Figure 1-3: Integrin signaling. Integrin cross-linking causes assembly of the FAK-SFK complex that also involves ILK, Cas, and Crk. Crk is involved in activation of both PI3K and Rac signaling, while FAK assists in PI3K activation. The PI3K and Rac pathways are independent, with PI3K signaling through Akt, and Rac signaling through Erk.

Actin remodeling

Actin remodeling is crucial to proper immune synapse (IS) formation and fusion of lytic granules with the plasma membrane. In this thesis I will focus on how actin remodeling is involved in NK cell IS formation - the study of which can provide additional potential targets for immunotherapy. Actin polymerization or formation of an actin plate at the immune synapse in NK cells was first described in 1983 [325]. Formation of the actin plate follows initial signaling from contact with the target. The canonical signaling pathway required for actin plate formation is initiated by receptor activation of Vav1 leading to a Wiskott–Aldrich Syndrome protein (WASP) → actin-related protein-2/3 (Arp2/3) pathway [326–328]. Formation of the actin plate is essential to NK cell mediated tumor cytotoxicity. Blocking actin polymerization with cytochalasin B greatly reduced the ability of NK cells to stay in contact with tumor targets [329]. The mechanism of how actin allows NK cells to remain in contact with tumor targets is not fully understood.

Polymerization of actin allows for NK cells to recognize the target by recruiting receptors and lipid rafts to the IS. Recruitment of receptors could be regulated by ezrin, radixin and moesin (ERM) family of proteins that are expressed at the NK IS [330]. These proteins link the cytoplasmic domains of receptors to the actin cytoskeleton, which traps those receptors and prevents their movement on the plasma membrane [331,332]. This results in an increase in receptor density at site with high levels of ERM family proteins. Recruitment of lipid rafts, containing activating receptors, to the IS is poorly described in NK cells [333]. However, disruption of lipid rafts, with the cholesterol sequestering drug

β-methylcyclo dextran (MBCD), reduced NK cytotoxicity. Recruitment of lipid raft to the IS in NK cells can be disrupted by inhibiting actin polymerization [334]. This suggests that actin mediated lipid raft recruitment to the NK IS is essential to NK cytotoxicity.

Disruption of lipid raft with MBCD also inhibits NK cell killing of *Cryptococcus [285]*. Actin has also been seen to polymerize at the synapse between NK cell-*Cryptococcus* [285]. The NK-cryptococcal conjugate also involves the formation of microvilli, which are formed by an actin-ERM protein dependent process [335,336]. These findings suggest that NK cells may require actin remodeling in order to kill *Cryptococcus*.

In tumor killing, actin accumulation at the IS is dependent on a WASP → Arp2/3 pathway [326]. One of the primary receptors responsible for actin remodeling against tumor targets is LFA-1 [337,338], however, since LFA-1 in not involved in cryptococcal killing [299], the cryptococcal signaling pathway responsible for actin remodeling is unknown.

In addition to NK cell activation and proper IS formation, actin remodeling is also involved in polarization of the MTOC toward the IS. The process of MTOC polarization is controlled by another group of actin nucleators called, formins (figure 1-4) [339]. Unlike Arp2/3, which is involved in actin branching [340], formins are required for the elongation of actin [341]. How formin activity causes polarization of the MTOC is unclear. In one model, formin activity nucleates the actin polymerization at the IS, and the polarization of the MTOC depends on minus-ended microtubule motor proteins

anchoring the microtubule to the actin cytoskeleton [342]. As the motor moves towards the minus end of the microtubule, they exert a pulling force on the MTOC. In T cells inhibiting dynein (minus end) motors reduced the speed of MTOC polarization [343]. Microtubules anchor to the actin network at the IS, so the activity of dynein would pull the MTOC towards the IS [343]. However, the formins - formin like-1 (FMNL1) and Dia1 - co-localize with the centrosome of T cells and allow MTOC polarization [344]. It is unclear why these formins localize at the centrosome, or how their presence at the centrosome assists in MTOC polarization.

The anti-cryptococcal signaling proteins - PI3K and Erk - can induce actin remodeling through both Arp2/3 and formins [345–348]. Taken together, Arp2/3 and formin are likely involved in NK-cryptococcal killing, and studying them can provide therapeutic targets.

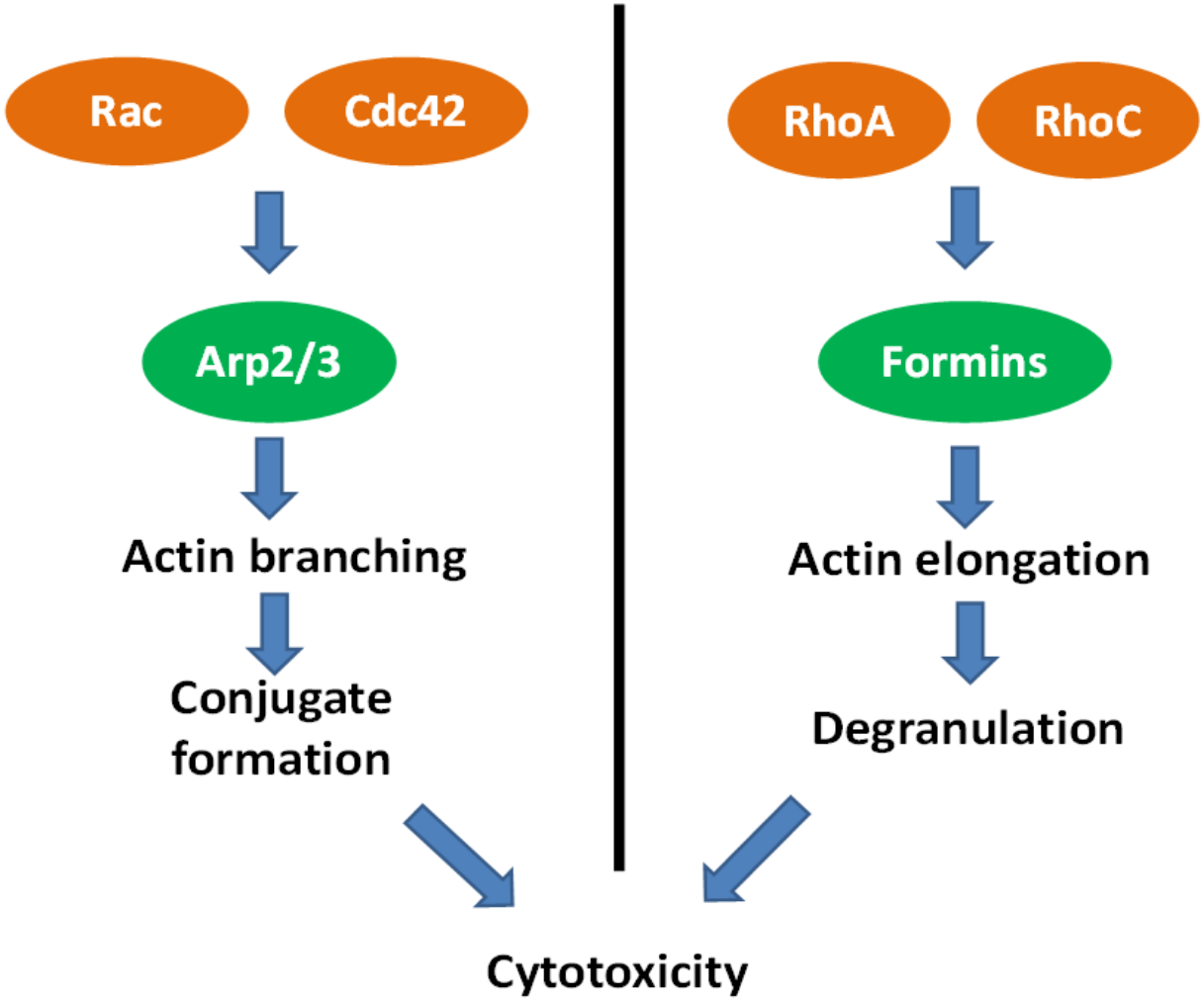


Figure 1-4: Role of actin remodeling in NK cytotoxicity. Arp2/3 and formins are both involved in actin remodeling. Arp2/3 is activated by Rac and Cdc42, while formins are activated by RhoA/C. Arp2/3 is required for actin branching, while formins are required for actin elongation. In NK cells, Arp2/3 and formins are both involved in cytotoxicity with Arp2/3 primarily facilitating conjugate formation, while formins allow for degranulation.

Atomic force microscopy

NK cell requires cell-cell contact in order to directly kill tumor, virally infected cells, and *Cryptococcus* [261]. Stable contact is maintained through maturation of the immune synapse. The strength of the immune synapse can be measured by the force required to separate the effector and target cells. A strong conjugate would require a stronger force to separate the cells. One way to measure the separation force is by atomic force microscopy (AFM). Many previous experiments have used AFM to determine cell-cell adhesion force - for example determining the binding force between promyeloblasts and human umbilical vein endothelial cells (HUVEC) [349], determining the force of single receptor-ligand interaction between 2 *Dictyostelium discoideum* cells [350], and determining the adhesion force between a melanoma cell line and HUVEC [351]. Using AFM to determine adhesion force has the advantage of measuring live cells in a physiologically relevant environment with high temporal and force resolution [351]. AFM involves a target cell attached to a flexible cantilever probe (figure 1-5). A laser is reflected off the probe, and used to track the bending of the probe. The probe is lowered onto the effector cells and contact causes the cantilever to bend away from the surface [352]. The bending of the cantilever deflects that laser at a different angle and is detected by a position sensitive photodiode [352]. Once a synapse has formed, force is applied to the cantilever to retract it. If there is an adhesion force between the target and

effector cells, then the cantilever will bend towards the surface and deflect the laser at a different angle. Once the retraction force is stronger than the adhesion force of the conjugate, there will be a large movement in the cantilever, as it snaps away from the effector cell and returns to its neutral conformation. By analyzing the bending of the cantilever, the retraction force on the cantilever, and distance the cantilever is from the surface, the adhesion force of the immune synapse can be determined.

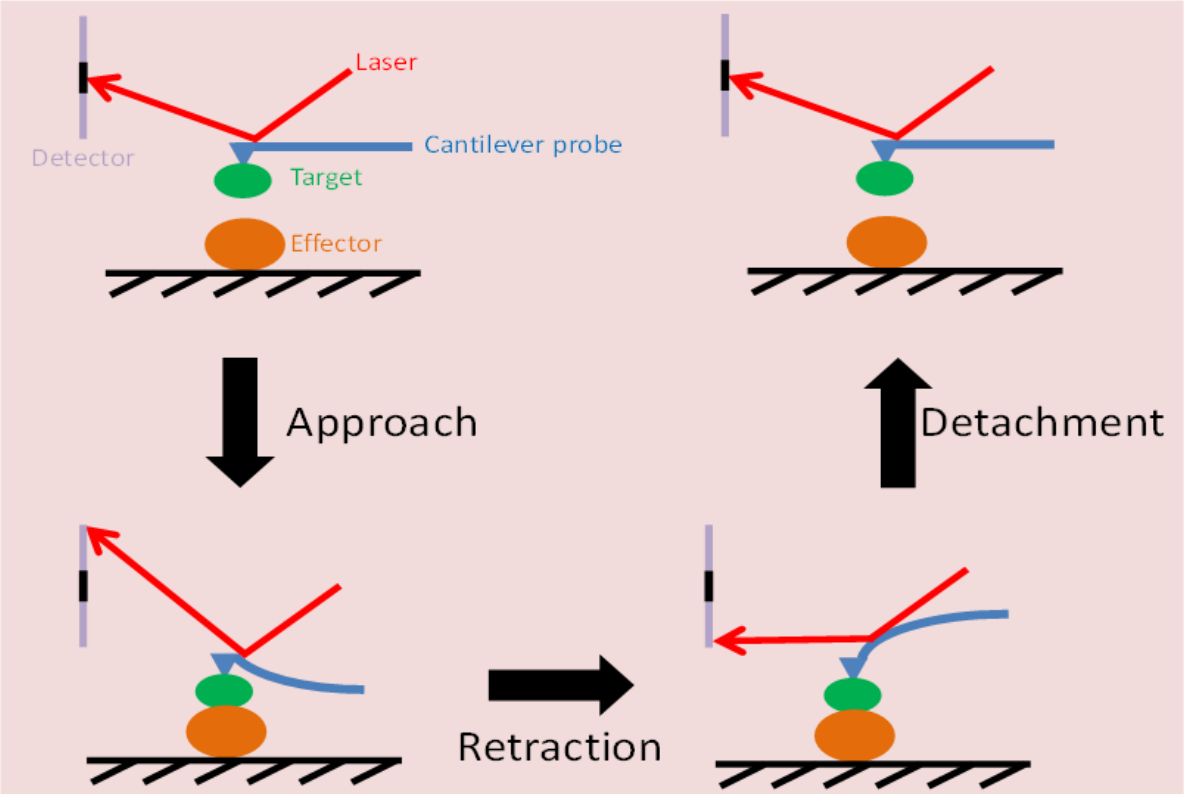


Figure 1-5: Diagram of atomic force microscopy. The target is attached to a flexible cantilever. A laser monitors the conformation of the cantilever. Lowering the cantilever against an effector cell causes the probe to bend away from the surface. During retraction the adhesion force between the effector and target will cause the cantilever to bend towards the surface. The adhesion force can be determined by the degree to which the probe bends before the target and effector separate from each other.

Concluding remarks and hypothesis

NK cell-mediated cryptococcal killing is poorly understood. NK cells are predominantly anti-tumor and anti-viral effector cells. Although NK cells possess anti-cryptococcal activity the signaling pathways involved are not fully understood. *Cryptococcus* activates a PI3K→ Erk signaling pathway, but the intermediate molecules involved are unknown. In tumor killing, PI3K can activate a Rac → PAK → MEK1/2 → Erk1/2 pathway [353,354]. Alternatively, PI3K can activate phospholipase C (PLC)γ and lead to Rac independent Erk activity [355,356]. Outside of Erk activation PLCγ and Rac activate different cytotoxicity pathways. PLCγ activates DAG and intracellular calcium signaling [357], while Rac regulates actin remodeling through WASP (Wiskott–Aldrich syndrome protein)-family verprolin homologous protein (WAVE) and Arp 2/3 [358]. Determining the involvement of PLCγ or Rac would aid in determining other signaling proteins involved in cryptococcal killing.

NK cell-mediated tumor killing is contact dependent and requires integrin binding. Integrin binding initializes immune synapse formation [359] and allows for activating NCRs to recognize their ligands. However it is unclear if integrins are involved in cryptococcal killing. Transwell experiments have demonstrated that NK cells need to contact *Cryptococcus* in order to facilitate killing [274]. Since contact is usually initiated by integrins, integrins are worth investigating. Against tumor targets, integrin signaling

directs granule polarization and allows for directional killing, while ITAM receptor signaling (such as through CD16) is required for granule fusion with the plasma membrane and degranulation [360]. Although a ITAM activating receptor, NKp30, has been identified in cryptococcal cytotoxicity, the integrins involved are unknown.

Actin remodeling is a crucial component to NK cell cytotoxicity, and allows for migration, immune synapse formation, and granule trafficking [361]. Given the importance of actin it is likely that actin remodeling proteins are required for cryptococcal killing. However, the role of Arp2/3 and formin have not been explored.

I hypothesize that Rac1 or PLCγ act as intermediates between PI3K and Erk, and that integrins, Arp2/3, and formins are required for NK mediated cryptococcal killing.

Chapter 2: Materials and Methods

2.1 Chemicals and Small Molecule Inhibitors

FITC was purchased from Sigma-Aldrich (St. Louis, MO, USA. Cat. #3326-32-7), EHT 1864 was purchased from Tocris (Bristol, United Kingdom, Cat. #3872). Rac inhibitor II was purchased from Millipore (Etobicoke, Ont, Canada. Cat. #553511). Ly294002 was purchased from Calbiochem (Etobicoke, Ont, Canada. Cat. #440202). Dasatinib was from New England BioLabs (Whitby, ON, Canada, Cat. #9052s). U73122 was obtained from Tocris (Cat #1268). CK666 was from Tocris (Cat. #3960). CPD22 was purchased from Millipore (Cat. #407331). SMIFH2 was purchased from Calbiochem (Cat. #344092). Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (Cat. #472301). PP2 was purchased from Enzo Life Sciences (Farmingdale, NY, USA, Cat. # BML-El297-0001). PP3 was purchased from Calbiochem (Cat. # 529574). Methyl β-cyclodextran (MBCD) was obtained from Sigma-Aldrich (Cat. #C4555). Carboxyfluorescein succinimidyl ester (CFSE) was obtained from Millipore (Cat. #4500-0270). Trypan blue stain 0.4% was purchased from Invitrogen Life Technologies (Burlington, ON, Canada, Cat. #15250). Cpd 22 purchased from EMD Millipore (Etobicoke, Ont., Canada, Cat. #407331). Poly-L-Lysine was purchased from Sigma-Aldrich (Cat. #P4707).

2.2 Antibodies

Protein bands in immunoblots were revealed with specific antibodies: rabbit polyclonal anti-phospho-AKT1/2/3 (Serine 473) (Santa Cruz, Dallas, Texas, USA, Cat. #sc79885R), mouse anti-Akt2 (Santa Cruz, Cat. #sc-81436), mouse anti-phospho-tyrosine (Millipore, Cat. #05-321), rabbit anti-phospho-Src family kinase (Y416) (Cell Signaling, Whitby, ON, Canada, 2101S), rabbit anti-phospho-Erk1/2 (T202/Y204) (Cell Signaling, 9101S), mouse anti-Erk1/2 (Cell Signaling, 9107S), rabbit Anti-pILK (ab1076, Millipore), mouse anti-ILK (clone 666.3, 05-1051, Millipore), mouse anti-Fyn (BD Transduction Laboratories, San Jose, CA, USA, 610163), mouse anti-Rac1 (Thermo Scientific, Waltham, MA, USA, 1862341), rabbit β-1 integrin (Cell Signaling, 4706S), goat anti-rabbit IgG infrared dye 700DX (Rockland, Limerick, PA, USA, 611-130-002), and goat anti-mouse IgG infrared dye 800 (Licor, Lincoln, Nebraska, USA, 923-32210). Cells for flow cytometry were labelled with specific antibody for mouse anti-CD11a PE-Cy5 (BD Biosciences, San Jose, CA, USA, 551131),rabbit anti-CD29 (Cell Signaling, 4706), mouse anti-CD29 conjugated to alexa fluro 488 (Biolegend, San Diego, CA, USA, 303015)

2.3 Cells and *Cryptococcus*

YT cells are an NK-like cell line isolated from the pericardial fluid of a 15 year old boy with acute lymphocytic leukemia [362]. YT cells possessed NK-like cytotoxic activity against a panel of target cells, including K562, T, and B cell lines [362]. Our YT cells were a gift from C. Clayberger, Emeritus Faculty, Stanford University, Stanford, CA, USA. YT cells were validated by its expression of NKp30 and NKp44, and lack of CD3.

YT cells were maintained in complete media, containing RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies), 1% non-essential amino acids (Invitrogen, Cat. #11140), 1% sodium pyruvate (Invitrogen, Cat. #11360), and 1% penicillin-streptomycin, in a 37°C 5% CO_2 incubator.

YT-Lifeact-RFP cells were created by transducing YT cells with a lentiviral p^{CMV}LifeAct-TagRFP virus (Ibidi, Martinsried, Germany, Cat. #60102). $1x10^4$ YT cells were transduced with the p^{CMV}LifeAct-TagRFP virus in RPMI 1640 media. 5% FBS was added to the cells 1 hour post-transduction. Cells were cultured overnight, and then transferred to complete media supplemented with 1µg/ml puromycin. Resistant cells were then sorted for high RFP expression using fluorescence-activated cell sorting (FACS). YT-Lifeact-RFP cells were maintained in complete media, in a 37°C 5% CO_2 incubator.

K562 were obtained from ATCC (Manassas, VA, USA, Cat. #CCL243). 721.221 cells were purchased from ATCC (Cat. #CRL1855). Both K562 and 721.221 cells were maintained in complete media, in a 37°C 5% CO_2 incubator. Primary NK cells were isolated from healthy donors using a NK isolation kit (Miltenyi Biotec, San Diego, CA, Cat. #130-092-657) as per manufacturer's instructions. Isolated cells were routinely >92% CD56+, <0.5%CD3+. *Cryptococcus neoformans* strain B3501 (ATCC, Cat. #34873) and strain 145 (ATCC, Cat. #62070) were grown to log phase in Sabouraud dextrose broth (Becton Dickinson (BD), Mississauga, ON, Canada, Cat. #238230) on a 32°C shaker overnight.

2.4 Immunoblotting

YT cells ($3x10^5$ to $3x10^6$) were preincubated with varying inhibitors for 1 hour in a 37°C CO_2 incubator. YT cells were then co-incubated with *C. neoformans* strain B3501 at and effector to target (E:T) ratio of 100:1 for varying time points in a 37°C water bath. Cells were lysed in Nonidet P40 (NP40) lysis buffer containing 50 mM Tris pH7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 1% NP40, and 0.02% NaN3. Lysis buffer was supplemented with phosphatase (Roche, Mississauga, ON, Canada) and protease inhibitors (Roche). Lysates were separated on a 4-12% Bis-Tris NuPAGE gels (Invitrogen, Cat. #NP0335BOX). After separation, samples were transferred to a nitrocellulose membrane and revealed with indicated antibodies. Bands were recorded using ODYSSEY infrared imaging system (Licor, Lincoln, Nebraska, USA). Densitometry was calculated by measuring the area under the intensity plot, using ImageJ (National Institute of Health (NIH), USA, Version 1.48). Fold increase in signaling compared to unstimulated was calculated as (intensity of stimulated condition normalized to loading control)/(intensity of unstimulated condition normalized to loading control)-1.

2.5 NK anti-cryptococcal killing assay

Cryptococcus neoformans strain B3501 and strain 145 were grown to log phase overnight in Sabouraud dextrose broth on an orbital shaker at 32°C. YT cells were co-cultured with the indicated strain of *C. neoformans* at an E:T ratio of 150:1 in round bottom 96-well plates, from Thermo Scientific (Cat. # 163320). CFU were determined at 24 and 48 hours post inoculation. The anti-cryptococcal activity of primary NK cells were

determined by co-culture with *C. neoformans* at an E:T ratio of 800:1 in round bottom 96-well plates. CFU were determined 24 hours post-inoculation. In experiments where EHT 1864, Rac inhibitor II, or MBCD were used, the inhibitors were added to the YT or primary NK cells at the same time that *Cryptococcus* was added. In addition, an equivalent volume of sterile H_2O was added to control wells to control for the highest levels of EHT1864 used and an equivalent concentration of DMSO was added to control for the highest levels of Rac inhibitor II used and PBS was added to control for MBCD. YT cells were pre-incubated with varying concentrations of U73122 for 1 hour, which has been shown to block lytic granule convergence in a similar NK cell line (YTS) [363]. YT cells were then washed with complete media, and incubated with *Cryptococcus* as described above. Primary NK cell and YT cell viability was determined by trypan blue staining. Percent viability was calculated as (number of trypan blue positive cells)/(total number of cells) x 100%. Interexperiemental statistics were calculated by: proportion of CA growth = average of [(mean CFU of a condition in a single experiment)/ (mean CFU of the *Cryptococcus* alone condition in a single experiment)]. Concentrations of inhibitors had minimal impact on viability of YT and primary NK cells.

2.6 Conjugate assay

In chapter 3: *C. neoformans* strain B3501 was labelled following the procedure for *C. gattii* as previously described by Huston et al [175]. Briefly, *C. neoformans* was cultured overnight to the exponential phase of proliferation and labelled with 2.5 µg/ml of fluorescein isothiocyanate **(**FITC) per 10^8 cells at 22°C for 10 minutes. *C. neoformans*

was then wash three times with phosphate buffered saline (PBS). YT cells or primary NK cells were co-incubated with 5 µl of anti-CD11a PE-Cy5 antibody and 100 µM EHT1864 or vehicle control for 30 minutes in a 37°C CO_2 incubator. YT cells or primary NK cells and different amounts of *Cryptococcus* were incubated together for 10 minutes at 37°C in 200 µl of complete media. YT cells or primary NK cells were then agitated by pipetting. Conjugates were detected by Guava EasyCyte flow cytometer (Cytosoft version 5.3, Guava technologies, Millipore, Danvers, MA), and data were analyzed by FlowJo software (Tree Star, Ashland, OR, USA). The percentage of NK cells in conjugates with *C. neoformans* were determined by: (number of green and red event)/(total number of red events) x 100%. For conjugate assays testing SFK, YT cells were pre-incubated with dasatinib for 60 minutes or PP2 or PP3, which served as the control for PP2 for 120 minutes in a 37°C CO_2 incubator. Other steps were performed the same as below.

In chapter 5: 721.221 cells were labeled with CFSE at 4uM for 10 minutes at room temperature. 721.221 cells and YT cells are co-incubated in a round-bottom 96 well plate for 90 minutes in a 37ºC CO_2 in the presence of various concentrations of CK666 or DMSO. YT cells are kept at a concentration of 150,000/ml in a volume of 200ul per well. The number of 721.221 cells were adjusted according to the E:T ratio. After incubation, dead cells were stained with 500ng/ml of 7-aminoactinomycin D (7-AAD) for 5 minutes. Cells were then spun down for 5 minutes at 600g and resuspended in complete media. Conjugates were analyzed by Guava EasyCyte flow cytometer

2.7 PBMC (Peripheral blood mononuclear cell) proliferation

PBMC were isolated from healthy adult donors - with no known history of cryptococcal infection - by Ficol-Paque gradient (GE Healthcare Life Sciences, Mississauga, ON, Canada, Cat. #17-1440-02). PBMC ($2x10^6$ per ml) were labeled with CFSE as per the manufacturer's instructions and stimulated with 5ug/ml of phytohaemagglutinin (PHA, Sigma-Aldrich) in the presence of 1uM, 2.5uM U73122, or DMSO control equivalent to the highest amount of U73122 used. PBMC were allowed to proliferate for 5 days in 24-well plates in 37°C CO_2 incubator. Levels of CFSE labeling were determined by Guava EasyCyte flow cytometer.

2.8 Rac-GTP precipitation

YT cells were pre-cultured with 10uM EHT 1864 or 100uM Rac inhibitor II or 50 µM Ly294002 or DMSO vehicle control for 1 hour at 37°C. YT cells were unstimulated or stimulated with *C. neoformans* for 4 minutes in a 37°C water bath. YT cells centrifuged at 6000g for 30 seconds and the supernatant was decanted. YT cells were then lysed and *Cryptococcus* was added to the unstimulated conditions in order to control for the additional volume of *Cryptococcus* in the stimulated conditions. Rac-GTP was then extracted according to manufacturer instructions with two modifications (Millipore, 17-10394). Protease inhibitor (Roche) was substituted for leupeptin, and 5ul of PAK's PBD conjugated magnetic beads was used. Aliquots of whole cell lysate were saved before the addition of PBD-coated beads, so that the total levels of Rac1 could be determined.

2.9 siRNA knockdown

In chapter 3: Two different sequences of siRNA specific against Rac1 (UAAGGAGAUUGGUGCUGUA and AUGAAAGUGUCACGGGUAA), one of which bears no resemblance to Rac2, and one non-targeting control sequence were purchased from Dharmacon RNAi Technologies (Lafayette, CO). Transfection of each of the siRNA sequences was performed by resuspending 3 million YT cells in 100 µl of Nucleofector solution (Kit V, Amaxa, Walkersville, MD) and adding 2 µg of the corresponding siRNA. The solution was then transferred to cuvettes and nucleofection performed using the O-017 program. A total of 500 µl of pre-warmed complete media was added and cells were transferred to 1.5 ml Eppendorf tubes and placed in a 37°C incubator for 10 minutes. Cells were then transferred to a 6-well plate containing 5 ml of pre-warmed complete media.

In chapter 4: siRNA specific against β-1 integrin's β chain (CD29) (GGAACCCUUGCACAAGUGA) were purchased from Invitrogen Life Technologies (Burlington, ON, Canada). siRNA against β-2 integrin was purchased from Thermo Scientific. Non-targetting siRNA was purchased from Cell Signaling (Whitby, ON, Canada). YT cells were transfected with 2ug of siRNA specific against β-1 integrin or β-2 integrin or control. Nucleofector kit V (Amaxa, Walkersville, MD) and Nucleofector II (Amaxa) was used to perform the nucleofection. Nucleofector program O-017 was used. YT cells were transferred into 500ul of pre-warmed complete media after transfection and then placed in a 37^{o}C incubator for 10 minutes. Cells were then transferred to 6

well plates and allowed to recover for 24 hours in a 37°C CO_2 incubator. The cells were then used in a killing assay as described below.

2.11 Atomic Force Microscopy

YT cells were pretreated with 10uM CK666 or DMSO control for 2 hour in a 37°C CO_2 incubator. YT cells were then placed into poly-L-lysine coated 35mm, low wall dishes from ibidi (Madison, Wisconsin, USA). 721.221 cells were labeled with 2uM CFSE for 15 minutes at room temperature. 721.221 cells or *C. neoformans* strain B3501 were fixed with 2% paraformaldehyde for 10 minutes at room temperature and then attach to tipless cantilevers (Novascan) by Cell-Tak (Corning, Cat: 354240). It is unlikely that paraformaldehyde fixation alters adhesion molecules involved since fixation of other receptors have retained morphology [364]. Adhesion forces were measured using a Zeiss (Oberkochen, Germany) Axiovert 200, with a JPK (Berlin, Germany) NanoWizard II. During the AFM experiment, cells were kept at 37°C and in a 5% CO_2 environment. Each cantilever was calibrated by lower the probe at 10μm/s at a relative set point of 0.2V and a contact duration of 0 seconds. The calibration force curve was used to manually calculate the Newtons/V and spring constant. Cells were contact with a force of 500pN for 10 to 60 seconds. At least 5 contacts were made between each effector-target pair. The strongest of the contacts was considered the adhesion force for that effector-target pair. Adhesion forces were analyzed using JPK NanoWizard Control version 4.2.28 and JPK Data Processing version spm-5.0.84.

2.12 Fluorescence Microscopy

YT Lifeact-RFP cells were preincubated with various inhibitors for 2 hours in complete media in a 37°C CO_2 incubator. 721.221 cells were labelled with 10uM carboxyfluorescein succinimidyl ester (CFSE) for 10 minutes at room temperature. YT Lifeact-RFP cells were then co-cultured with 721.221-CFSE or *C. neoformans* strain B3501 for 1 hour at an E:T ratio 1:1 in complete media in a 37°C CO_2 incubator. Cells were co-cultured in a 24 well plate (Corning, Cat. #3526) that had 24mm glass cover slips at the bottom. Concentration of YT Lifeact-RFP cells varied from 80,000 per condition to 150,000 per condition. After the 1 hour incubation, cover slips were removed from the wells and washed with PBS. Cells were then fixed with 2% paraformaldehyde for 10 minutes at room temperature. Cover slips were wash with PBS again, and then mounted onto glass slides with ProLong® Gold Antifade Mountant with DAPI (Thermo Fisher, Cat. #P36931).

Slides were imaged using a Zeiss laser scanning microscope 780, with a 63x oil objective (Zeiss Plan Apochromat, NA 1.4). Zen version 2.1 (Zeiss) software was used to capture the images.

2.13 Microscopy image analysis

The maximum intensity projection of the actin channel for each image was calculated use Zen version 2.1, and then exported as .tif files. The locations of the center of the YT cell and the IS were determined manually for each image. A line was then drawn from the IS to the center of the YT cells, and then different lines were drawn from the center of the YT cell to each pixel with intensity in the actin above a threshold

10% of the dynamic range. The angle that is formed at the center of the YT cells by the line from the IS and the line to the actin pixel was used to determine if that actin pixel was on-target, off-target, or ignored. If the angle is less than 35 degrees, then the actin pixel was considered on-target. If the angle is greater than 35 degree, but less than 70 degrees, then the actin pixel was considered off-target. If the angle was greater than 70 degrees, then the actin pixel was ignored for further calculations. The sum of the intensity of the on-target actin pixels was divided by the sum of the intensity of off-target actin pixels. If actin was evenly distributed throughout a spherical cell, then a ratio of 1 would be expected. The program used to perform this analysis was written in R language version 3.2.0 [365] using RStudio version 0.98.1091 [366] with libraries from EBImage [366,367]. Source code can be found in appendix B.

2.14 Statistics

GraphPad Prism was used to evaluate statistics. Error bars represent the standard error of the mean (SEM). Data were analyzed by one-way analysis of variance (ANOVA) with Bonferroni correction. $P<0.05$ is considered to be a statistically significant different between conditions. Percent reduction in conjugates compared to H_2O or DMSO was analyzed by the column statistics program in GraphPad Prism.

2.15 Ethics

Experimental protocols were approved and performed following the guidelines from the Conjoint Health Research Ethics Board of the University of Calgary, protocol number REB15-0600.

Chapter 3: Rac1 signaling in anti-cryptococcal killing

3.1 Contributions

This chapter is adapted from the manuscript:
Ras-related C3 Botulinum Toxin Substrate (Rac) and Src Family Kinases (SFK) are Proximal and Essential for Phosphatidylinositol-3 Kinase (PI3K) Activation in Natural Killer (NK) Cell-Mediated Direct Cytotoxicity Against *C. neoformans*
Richard F. Xiang, Danuta Stack, Shaunna M. Huston, Shu Shun Li, Henry Ogbomo, Stephen K. Kyei, and Christopher H. Mody

Some of the purifications of NK cells from whole blood were done by Danuta Stack. In some experiments, collection of blood was performed by Shaunna M. Huston. Shu Shun Li, Henry Ogbomo, and Stephen K. Kyei helped to passage and maintain YT cells and *Cryptococcus neoformans*.

3.2 Introduction

NK cells require PI3K mediated activation of Erk in order to clear *C. neoformans*, however the intermediate molecules are unknown. The intermediate proteins belong to two pathways causing either actin remodeling or calcium signaling and determining which pathway is involved further our understanding of direct anti-cryptococcal

cytotoxicity. Rac1 - a protein involved in actin remodeling - is also required for receptor-mediated NK anti-tumor signaling in a PI3K → Rac1 → Erk pathway [368]. Against Raji tumor targets, the human NK cell line, NK92 and primary NK cells required Rac1 to initiate a PAK1 → mitogen activated protein kinase kinase (Mek) 1/2 → Erk1/2 signaling cascade that allows for cytotoxic granule mobilization and release [368]. Rac1 activation was found to be dependent on PI3K, since NK92 cells transfected with constitutively active Rac1 were able to continue killing tumor targets in the presence of PI3K inhibitors, whereas control cells were not [368].

PI3K can also activate Erk via phospholipase C gamma (PLCγ) [369–373]. In certain cells, such as platelets, PLCγ activity is also activated by PI3K [372]. PLCγ activity is also required for the activation of cytokine mediated Erk signaling[369]. Similar to Rac1, PLCγ is also essential in NK anti-tumor cytotoxicity [371]. However, it is not clear whether Rac1 or PLCγ participate in NK cell microbial killing and where they are positioned in the signaling cascade. In this chapter I will examined the pathway used in NK cell-mediated cryptococcal killing by investigating both PI3K→ Rac1→ Erk and PI3K → PLCγ → Erk pathway.

Tumor and viral ligand activated NK cell signaling pathways have multiple points of convergence and divergence. For example, natural-killer group 2 member D (NKG2D) and 2B4 are two NK cell receptors that initiated different signaling pathways; YINM, or immunoreceptor tyrosine-based switch motif (ITSM) signaling respectively [374]. Although two different pathways were initiated, both pathways converged into a

Vav1→PLCγ pathway that led to degranulation [374]. In cryptococcal killing the SFK→ PI3K→ Erk cytotoxicity pathway has been identified [375,376]. I considered the possibility that multiple anti-cryptococcal signaling pathways converge onto this central pathway. Since Rac1 and PLCγ are activated by PI3K and led to Erk signaling, convergence of Rac1 and PLCγ could be required for NK-cryptococcal killing [369,373,377]. By studying Rac1 and PLCγ, this study aims to elucidate the interconnections between the pathways that are activated by *Cryptococcus*.

To examine signaling in NK cell-mediated cryptococcal killing, I used the human NK cell line, YT, and primary blood-derived NK cells isolated from healthy adults. Rac1 activation was assessed by a Rac1 pull down assay. This assay involving precipitation with the p21 Binding Domain (PBD) of PAK. The PBD of PAK directly binds to the active (GTP) form of Rac1. Therefore, by "pulling down" with PBD coated beads, I could extract the active levels of Rac1-GTP.

The requirement for Rac1 in the anti-cryptococcal activity of NK cells was assessed using two independent pharmacologic inhibitors and siRNA knockdown, and examining cryptococcal colony forming units (CFU). A flow cytometric assay was used to assess conjugate formation between NK cells and *Cryptococcus*. To determine the proximal and distal signaling elements, the interplay between Rac1, SFK, PI3K, and Erk1/2 was assessed using loss of function approaches, and performing immunoblots for activation of each element.

3.3 Results

Rac1, in NK cells, is activated by cryptococcal stimulation

Both NK cell tumor and cryptococcal killing depend on a PI3K → Erk signaling pathway

[378][379]. PI3K can activate Erk by Rac1 or PLCγ intermediates [379–381]. To determine if cryptococcal killing depends on Rac1 or PLCγ, I first investigated Rac1 activation in YT cells. I stimulated YT cells with *C. neoformans* at varying time points, and found that cryptococcal stimulation activated Rac1 (Rac1-GTP) as assessed by binding to PAK's PBD conjugated to magnetic beads (Figure 3-1). Rac1 activation occurred by 5 minutes, and returned to baseline after 15 minutes. Thus, YT cells responding to cryptococcal stimulation activated Rac1, suggesting that Rac1 may play a role in anti-cryptococcal signaling.

PLCγ is not required for NK cell anti-cryptococcal activity

I also investigated PLCγ by pre-treating YT cells with the PLCγ inhibitor, U-73122, at concentrations found to inhibit tumor killing [382]. The anti-cryptococcal activity was examined and I found that the addition of YT cells significantly reduced the number of cryptococcal CFU, which is consistent with our previous observations of killing [376,383,384]. Despite using concentrations of U-73122 in excess to inhibit PLCγ, there was no impact on YT anti-cryptococcal killing, suggesting that PLCγ was not involved in NK cell-mediated cryptococcal killing (Figure 3-2). This lack of inhibition was not due to inactivity of U-73122, since a lower concentration of the inhibitor was capable of blocking proliferation of PBMC (Figure 3-2).

Rac1 is required for NK cell killing of *Cryptococcus*

Having determined that Rac1 is activated by *Cryptococcus* I explored whether Rac1 is required for NK cytotoxicity by using two different inhibitors of Rac1, EHT 1864

and Rac inhibitor II. EHT 1864 binds to Rac1 and facilitates the release of GTP, preventing the association of Rac1 with downstream effectors[385]. By contrast, Rac inhibitor II prevents Rac1 activation by blocking the association of Rac1 with GEF[386]. To confirm that the Rac1 inhibitors blocked Rac1 activation, YT cells were pre-treated with both Rac1 inhibitors and stimulated with *Cryptococcus.* Immunoblotting confirmed that both inhibitors successfully reduced the levels of Rac1-GTP (Figure 3-3). YT cells were co-incubated with two different strains of *C. neoformans* in the presence of varying concentrations of EHT1864, Rac inhibitor II, or DMSO vehicle control. I found that the addition of YT cells to *C. neoformans* cultures reduced the number of CFU for both B3501 strain and strain 145 (Figure 3-4). Treatment of YT cells with EHT1864 or Rac inhibitor II caused a dose dependent increase in CFU compared to vehicle controls, indicating a loss of anti-cryptococcal activity (Figure 3-4). Trypan blue viability staining showed that the difference in viability between EHT 1864, Rac inhibitor II, and DMSO treated cells was less than 10%. These results suggest that Rac1 was required for NK cell-mediated killing of *Cryptococcus.*

Studies were performed to determine whether Rac1 was required for primary NK cells to kill *Cryptococcus.* Primary NK cells were isolated from healthy donors. Primary NK cells were rested overnight and co-cultured with *C. neoformans* strain B3501, in the presence of EHT1864 or control. I found that EHT 1864 inhibited the anti-cryptococcal killing of primary NK cells (Figure 3-5), suggesting that the importance of Rac extended to primary NK cells.

Despite using two different pharmacological inhibitors that work by two different mechanisms, I considered the possibility that a non-specific pharmacologic effect

accounted for the reduced anti-cryptococcal activity. To exclude this possibility, experiments were performed using Rac1 siRNA knockdown. Two different sequences of Rac1 siRNA were used to knockdown Rac1 expression in YT cells in order to control for off target effects. Immunoblotting revealed that both sequences of siRNA reduced Rac1 expression, while the non-targeting siRNA did not (Figure 3-6). I observed that YT cells, in which Rac1 was knocked down, lost cytotoxic potential against *C. neoformans* compared to mock or non-targeting siRNA transfected YT cells (Figure 3-6), confirming that NK anticryptococcal activity was Rac1 dependent.

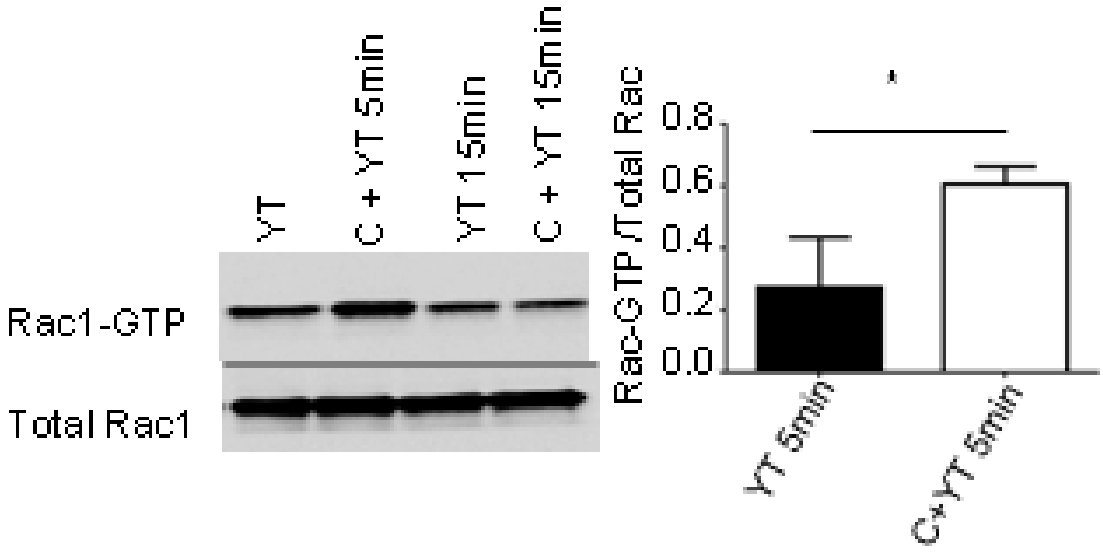


FIGURE 3-1. Rac is activated by cryptococcal stimulation
Left: YT cells were stimulated with *Cryptococcus* and levels of Rac-GTP were measured by immunoblotting. Right: Densitometry of normalized Rac-GTP/Total Rac of the mean of 3 experiments. * $p<0.05$. Error bars represent standard error. C+YT: YT co-cultured with *Cryptococcus.* YT: Unstimulated YT cells.

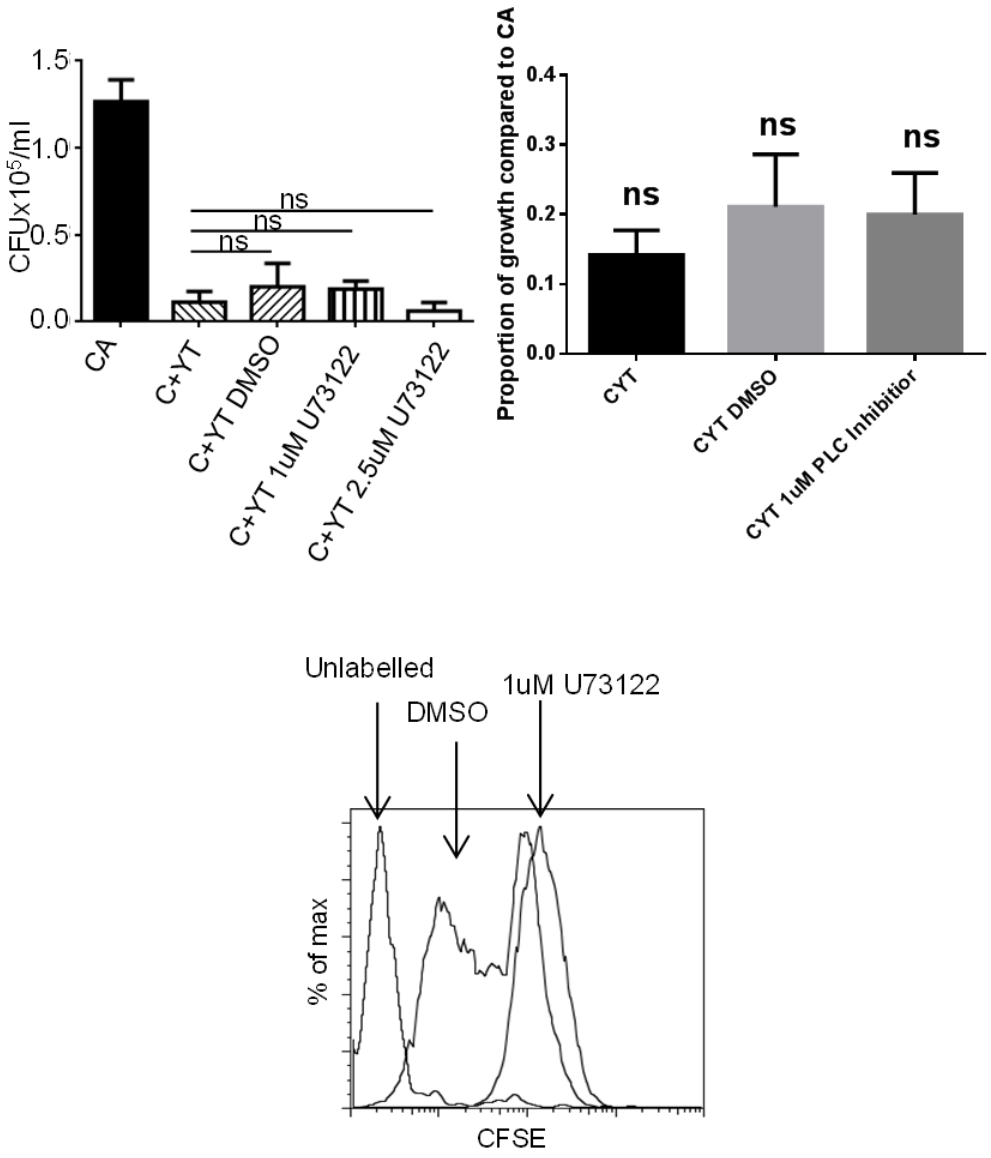


Figure 3-2: PLCγ is not required for cryptococcal killing. Top-left: YT cells were pre-incubated with U73122 or DMSO control, and co-cultured with *Cryptococcus* overnight. Bars show the mean of the CFU from quadruplicate wells in a single experiment. Error bars are the SEM. Data are representative of 3 experiments. Top-right: Inter-experimental statistics of 3 experiments of cryptococcal growth compared to *Cryptococcus* alone. Bottom: PBMC were labelled with CFSE and stimulated with PHA for 5 days in the presence of DMSO or U73122. A reduction in fluorescence intensity indicates proliferation. Data are representative of 3 experiments. Each experiment used PBMC from a different donor isolated on a different day. ns: non-significant. C+YT: YT co-cultured with *Cryptococcus.* CA: *Cryptococcus* alone.

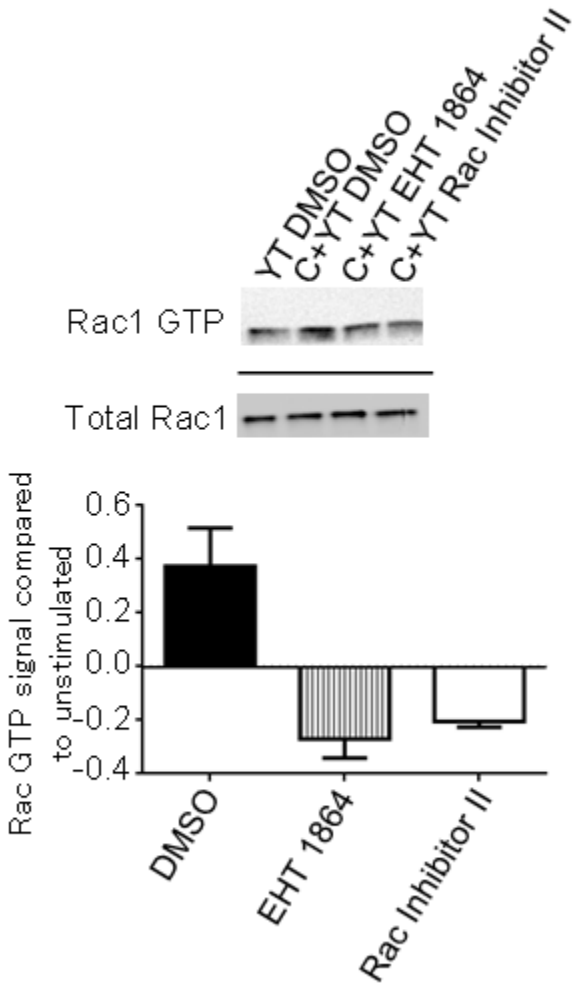


Figure 3-3: Rac inhibitors reduced cryptococcal activation of Rac. YT cells were pre-incubated with EHT 1864 or Rac inhibitor II and stimulated with *C. neoformans*. Levels of Rac-GTP were determined. Data are representative of 2 experiments. C+YT: YT co-cultured with *Cryptococcus.* YT: Unstimulated YT cells.

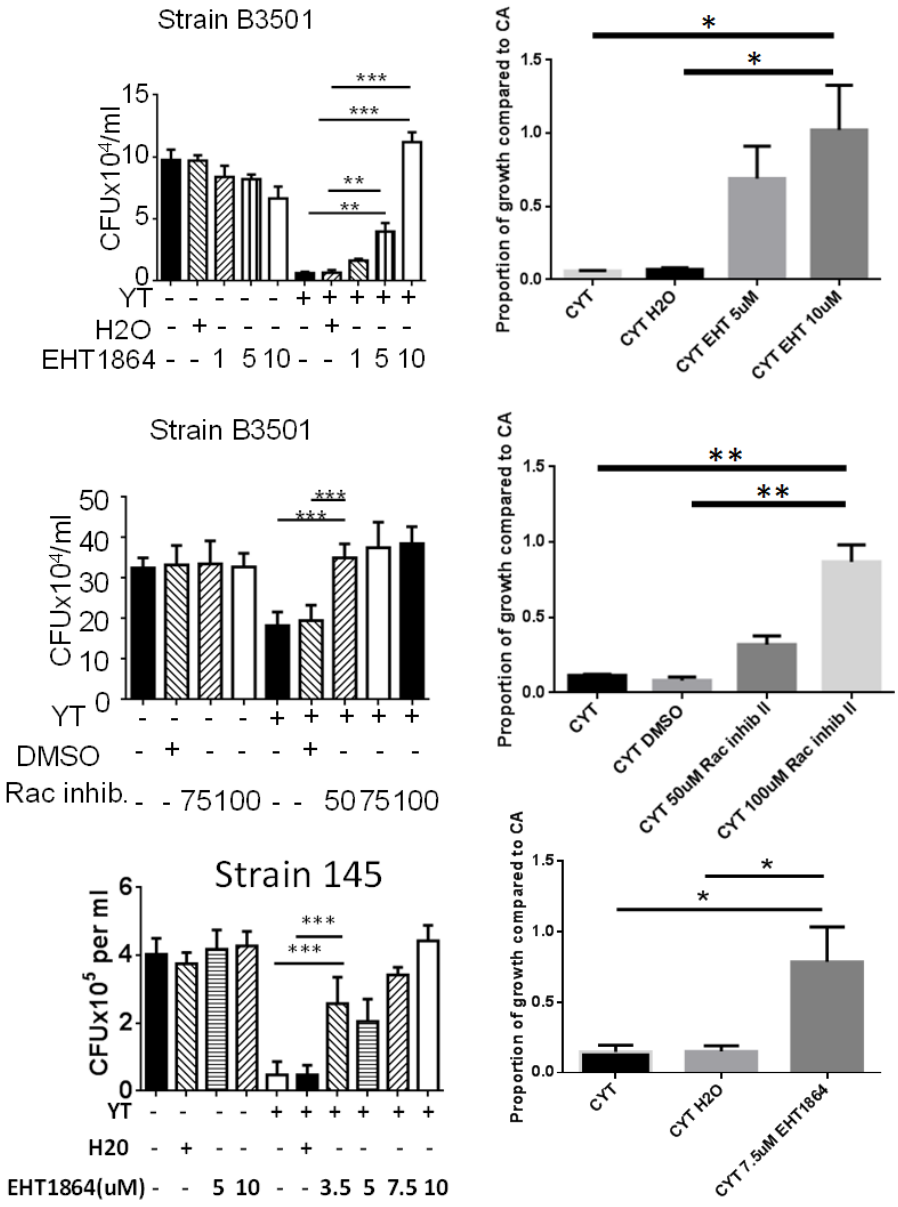


Figure 3-4: Rac is required for NK mediated cryptococcal killing. YT cells were co-incubated with *Cryptococcus* strain B3501 (top and middle) overnight in the presence or absence of EHT 1864 (top) or Rac inhibitor II (middle). YT cells were also co-incubated with strain 145 (bottom) in the presence and absence of EHT 1864. Left: Data from a single experiment that is representative of 3 independent experiments. Right: Inter-experimental statistics from 3 experiments. Concentrations are in μM. *p<0.05 **p<0.01 ***p<0.001. Error bars represent SEM.

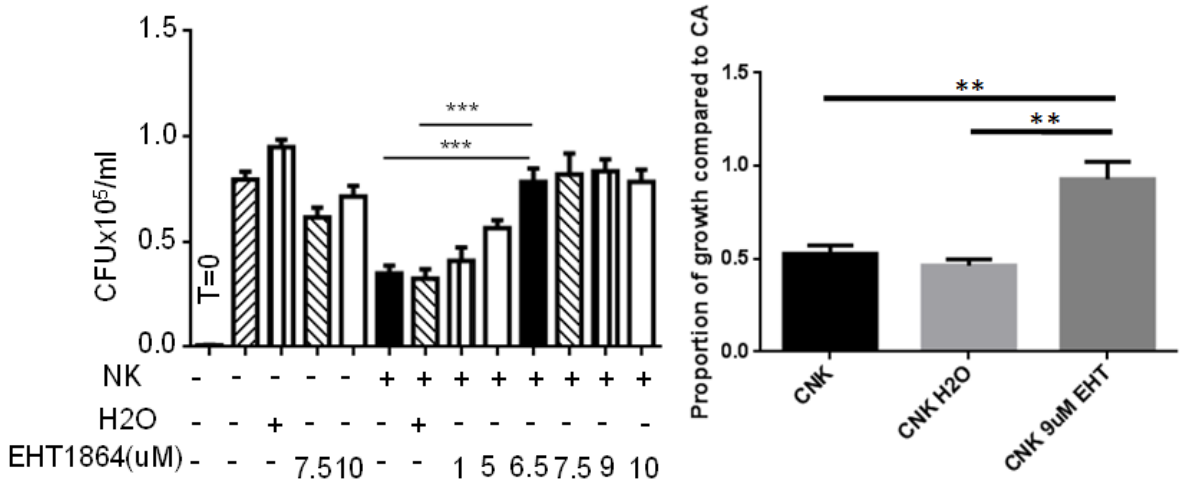


Figure 3-5: Primary NK cells require Rac to kill *Cryptococcus.* Left: Primary NK cells were co-cultured with *Cryptococcus* in the presence of EHT 1864 or H_2O control. Left-most condition shows the CFU before the 24 hour incubation. Data is representative of 3 independent experiments. Each experiment used NK cells from a different donor isolated on a different day. Right: Inter-experimental statistics from 3 experiments. ***p<0.001. Error bars represent SEM.

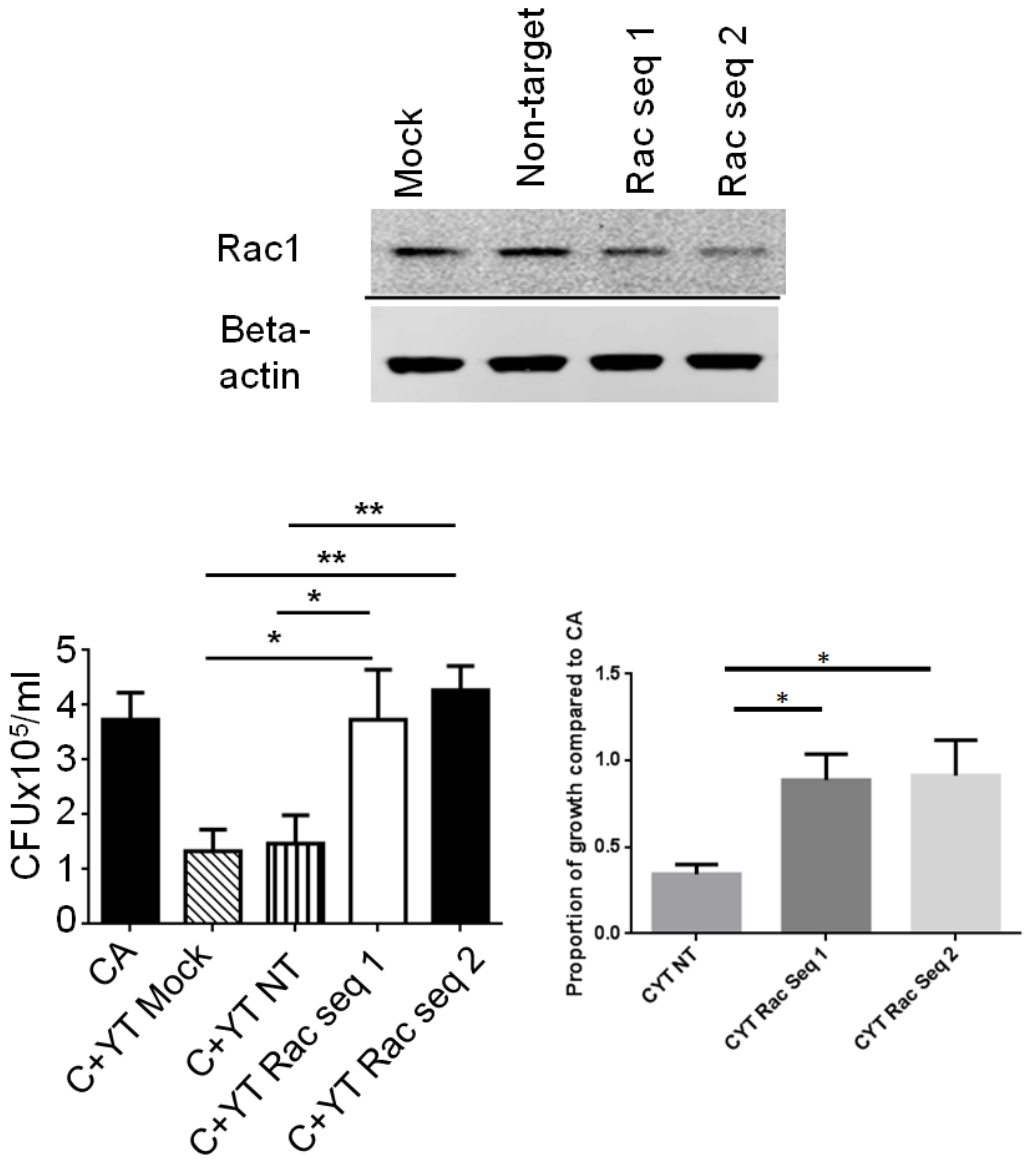


Figure 3-6: siRNA knockdown of Rac1 inhibits NK cell killing of *Cryptococcus*. Top: Levels of Rac1 in transfected YT cells were analyzed by immunoblotting. Bottom Left: YT cells were transfected with non-targeting siRNA or two different sequences of Rac1 specific siRNA. Transfected cells were used in a killing assay with *C. neoformans*. Data were representative of 3 experiments. Bottom Right: Inter-experimental statistics from 3 experiments. * $p<0.05$ ** $p<0.01$ *** $p<0.001$. Error bars represent SEM. C+YT: YT co-cultured with *Cryptococcus.* CA: *Cryptococcus* alone.

Conjugate formation between *Cryptococcus* and NK cells is Rac independent

Having demonstrated that Rac was required in cryptococcal killing, I hypothesized that Rac would affect conjugate formation, similar to its role against K562 targets [387]. I tested conjugate formation between NK cells and *Cryptococcus* by labeling YT cells with anti-CD11a conjugated to PE-Cy5 (since CD11a has previously been shown to be uninvolved in conjugate formation or killing), and *Cryptococcus* with fluorescein isothiocyanate (FITC). This allowed conjugate formation to be analyzed by flow cytometry as double positive events, as previously described [388]. I found that increasing the proportion of *Cryptococcus* increased the ratio of YT cells that formed conjugates (Figure 3-7). Despite the observation that EHT 1864 abrogated anti-cryptococcal activity, when I inhibited Rac activity with EHT 1864, surprisingly, I found no difference in conjugate formation. As a positive control I tested if disruption of lipid rafts would prevent conjugate formation and found that inhibiting lipid raft formation with methyl β-cyclo dextrin (MBCD) modestly, but significantly reduced conjugate formation and inhibited NK cell killing of *Cryptococcus* (Figure 3-8). These observations suggested that, unlike the requirement for lipid rafts, Rac was not involved in the formation of the conjugates between NK cells and *Cryptococcus*.

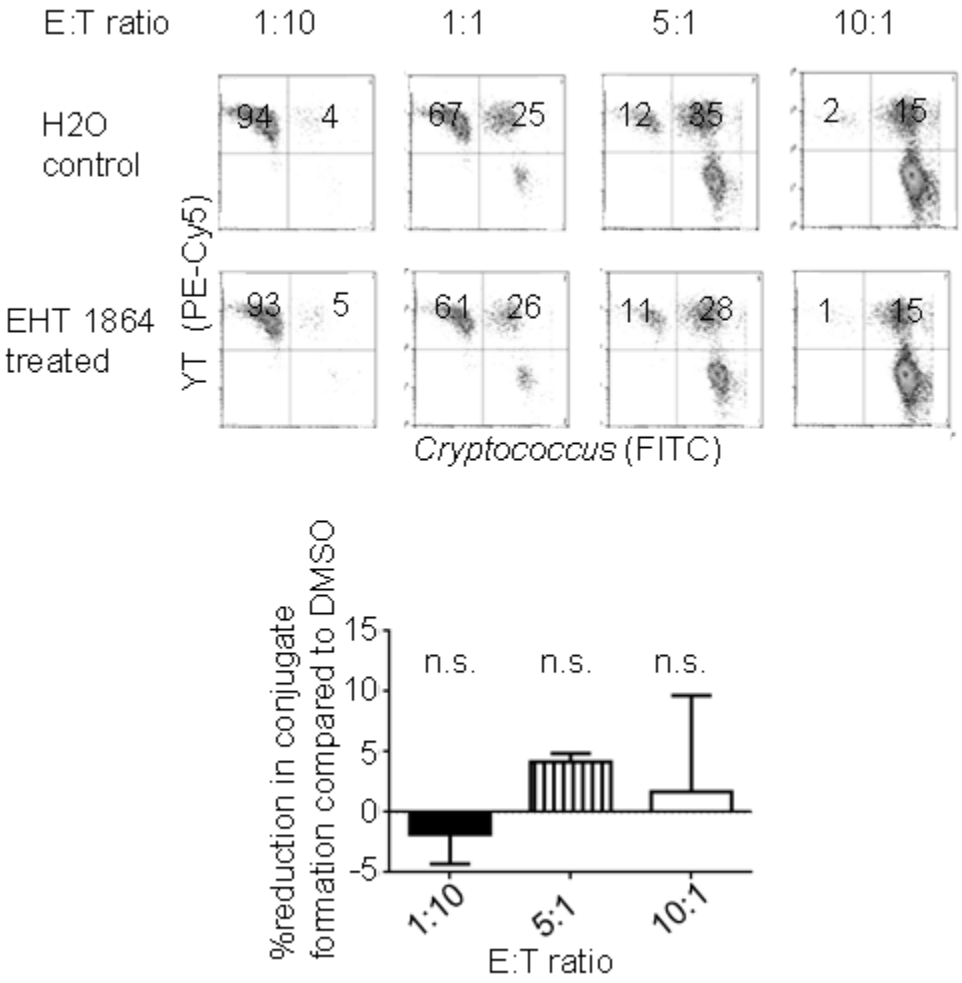


Figure 3-7: Rac is not required for NK-cryptococcal conjugate formation. YT cells were labelled with anti-CD11a conjugated to PE-Cy5 in the presence of 10uM EHT1864 or H_2O control. *C. neoformans* was labelled with FITC. YT cells and *Cryptococcus* were then co-incubated together at varying effector to target (E:T) ratios. Conjugates were analyzed by flow cytometry. Top: Events that matched the profile of YT cells by forward and side scatter were analyzed for the presence of those that had bound FITC labelled *Cryptococcus*. Bottom: Percentage of cells forming conjugates was calculated by (number of green and red events)/(total number of red events). % reduction in conjugate formation compared to H_2O was calculated by: (1-(% conjugates in EHT 1864 treated)/(%conjugates in H2O))*100%. Data are representative of 3 experiments. n.s. No statistically significant difference compared to H_2O.

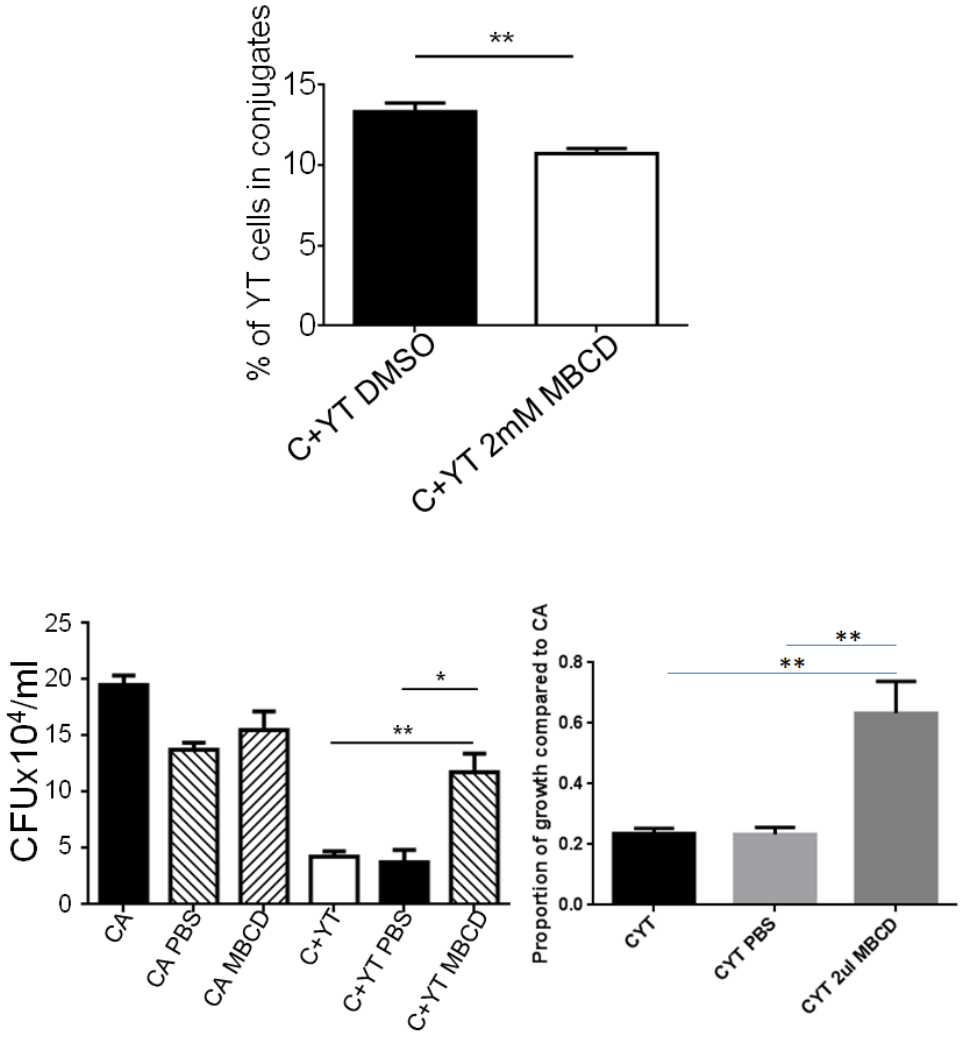


Figure 3-8: Lipid raft disruption is required for conjugate formation and cytotoxicity. Top: MBCD treatment of NK cells reduces conjugate formation at E:T ratio of 1:3. YT cells were labelled with anti-CD11a conjugated to PE-Cy5 and either EHT1864 or DMSO control. *C. neoformans* was labelled with FITC. YT cells and *Cryptococcus* were then co-incubated together at 1:3 E:T ratio. Conjugates were analyzed by flow cytometry. Bar graph depicts the mean of three separate experiments ± SEM. Bottom Left: YT cells were co-incubated with *Cryptococcus* in the presence of MBCD or PBS. CFU was counted at 24 hours. Data are representative of 3 experiments. Bottom Right: Inter-experimental statistics from 3 experiments. * $p<0.05$. ** $p<0.01$. C+YT: YT co-cultured with *Cryptococcus.* CA: *Cryptococcus* alone.

Erk mediated anti-cryptococcal activity is Rac-dependent

Having demonstrated that Rac was required for NK cell killing but not for NK cell-cryptococcal conjugate formation, I examined if Rac1 is involved in the PI3K → Erk cytotoxic pathway. Previous studies showed that Erk was essential in both NK cell-mediated tumor and microbial killing, and that Rac1 was required to activate Erk in tumor killing [373,375]. Since Rac1 and Erk are important in cryptococcal killing, I examined if Rac also activated Erk in response to *Cryptococcus.* I treated YT cells with EHT 1864 or Rac inhibitor II, and then stimulated them with *C. neoformans.* Both Rac inhibitors reduced *Cryptococcus* dependent Erk phosphorylation (Figure 3-9). I also knocked down the expression of Rac1 in YT cells and found that the reduction of Rac1 expression reduced phosphorylation of Erk in response to cryptococcal stimulation (Figure 3-10). Both knockdown and inhibitor data suggest that Rac activity was necessary for Erk activation in response to *Cryptococcus*.

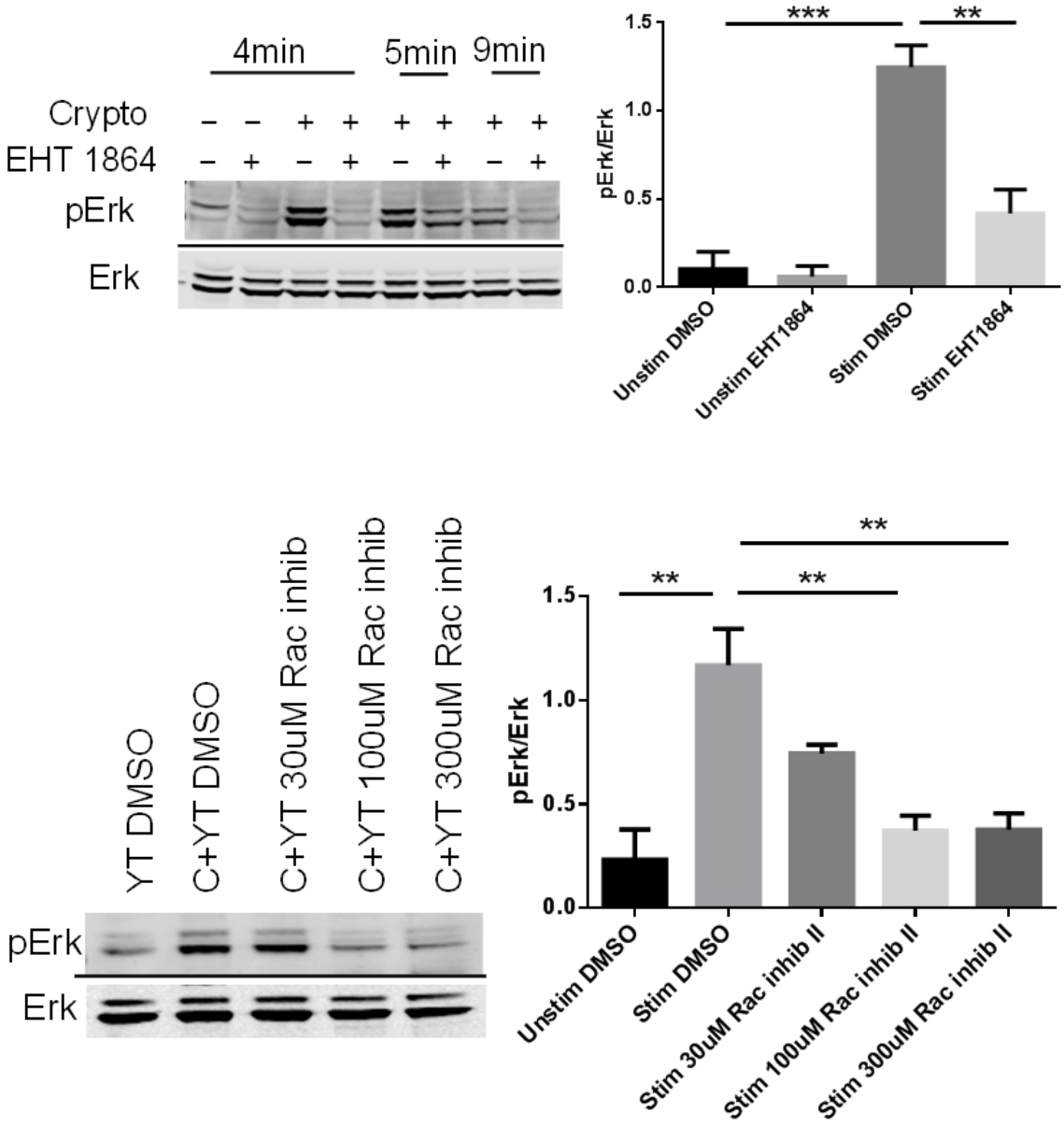


Figure 3-9: Rac small molecule inhibitors inhibit cryptococcal activation of Erk in NK cells. Top left: YT cells were preincubated with the Rac inhibitor EHT 1864, and then stimulated with *C. neoformans* for the indicated times. YT cells were then lysed and immunoblotted for pErk and Erk. Top right: Densitometry of Erk phosphorylation at 4 minutes. Bars are means of 3 experiments ± SEM. Bottom left: YT cells were preincubated with various concentrations of Rac inhibitor II. YT cells were then stimulated with *Cryptococcus* and lysed. Lysates were immunoblotted for pErk and Erk. Bottom right: Densitometry of Erk phosphorylation. Bars are means of 3 experiments ± SEM. ** p<0.01, *** p<0.001

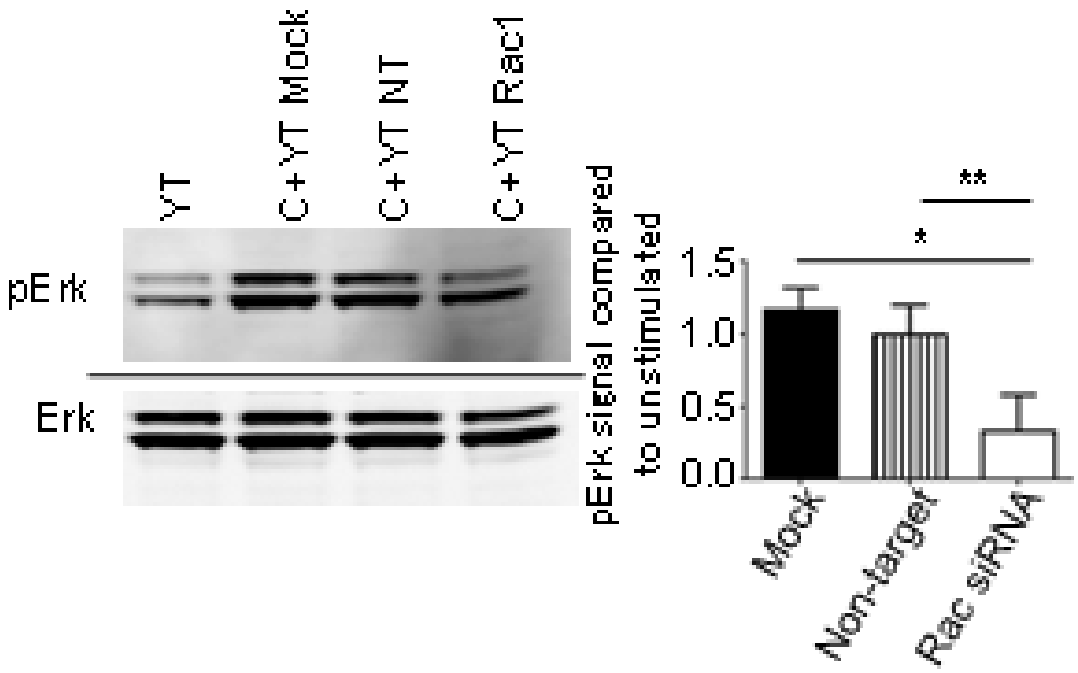


Figure 3-10: YT transfected with non-targeting or Rac1 specific siRNA inhibits Erk activation. Transfected YT cells were stimulated with *Cryptococcus* and levels of pErk and Erk were determined by immunoblots. Immunoblot is representative of 3 experiments. Error bars represent SEM. YT: Unstimulated YT cells alone. C+YT: YT cells stimulated with *Cryptococcus***.** NT: non-targeting. * $p<0.05$. ** $p<0.01$

PI3K anti-cryptococcal activity is Rac1 dependent

Since Rac1 was required for activation of Erk in NK cells stimulated by *C. neoformans,* and previous studies found that PI3K was also responsible for Erk activation [375], I sought to determine the relationship between Rac1 and PI3K. In NK cell-mediated tumor killing, PI3K activates Rac1, which in turn activates Erk [373]. Activation of Rac1 by PI3K is shared in numerous cell types including: endothelial cells, T cells, and neutrophils [389]. However, other studies raised the possibility of a different pathway where Rac1 may be proximal to PI3K, thus regulating PI3K activity [390]. To investigate the interaction between PI3K and Rac1 I pretreated YT cells with EHT 1864 and assessed Rac-α serine/threonine-protein kinase (Akt) phosphorylation, which is a commonly used as a surrogate for PI3K activity in response to stimulation with *C. neoformans* [375,391]. Rac1 inhibition reduced Akt phosphorylation in response to *Cryptococcus* (Figure 3-11). Additionally, YT cells transfected with Rac1 siRNA had reduced activation of Akt when stimulated by *Cryptococcus* (Figure 3-12). Together, these data indicate that unlike the signalling pathway in tumor cytotoxicity, Rac1 was proximal and required for PI3K activation.

Although Rac1 was required for activation of PI3K, I considered the possibility that PI3K and Rac operated in a self-amplifying loop in which PI3K also activated Rac1, as occurs in the leading edge of migrating neutrophils [392]. To test this possibility, PI3K activity was inhibited by pre-treating YT cells with Ly294002. YT cells were then stimulated with *C. neoformans* for 5 minutes, and the level of active Rac1 was determined by precipitation with PAK PBD. I found that inhibition of PI3K activity did not affect *Cryptococcus* induced Rac1 activation (Figure 3-13). To ensure that PI3K was

adequately inhibited, I performed immunoblots to examine the activation of Akt in the presence of Ly294002. I found that Ly294002 significantly inhibited phosphorylation of Akt, suggesting that PI3K activity is sensitive to Ly294002 (Figure 3-14). This datum agrees with previous publications where Ly294002 also inhibited PI3K activity in YT cells [375]. Therefore, my data indicate that Rac1 was proximal to PI3K and not in a self-amplifying loop.

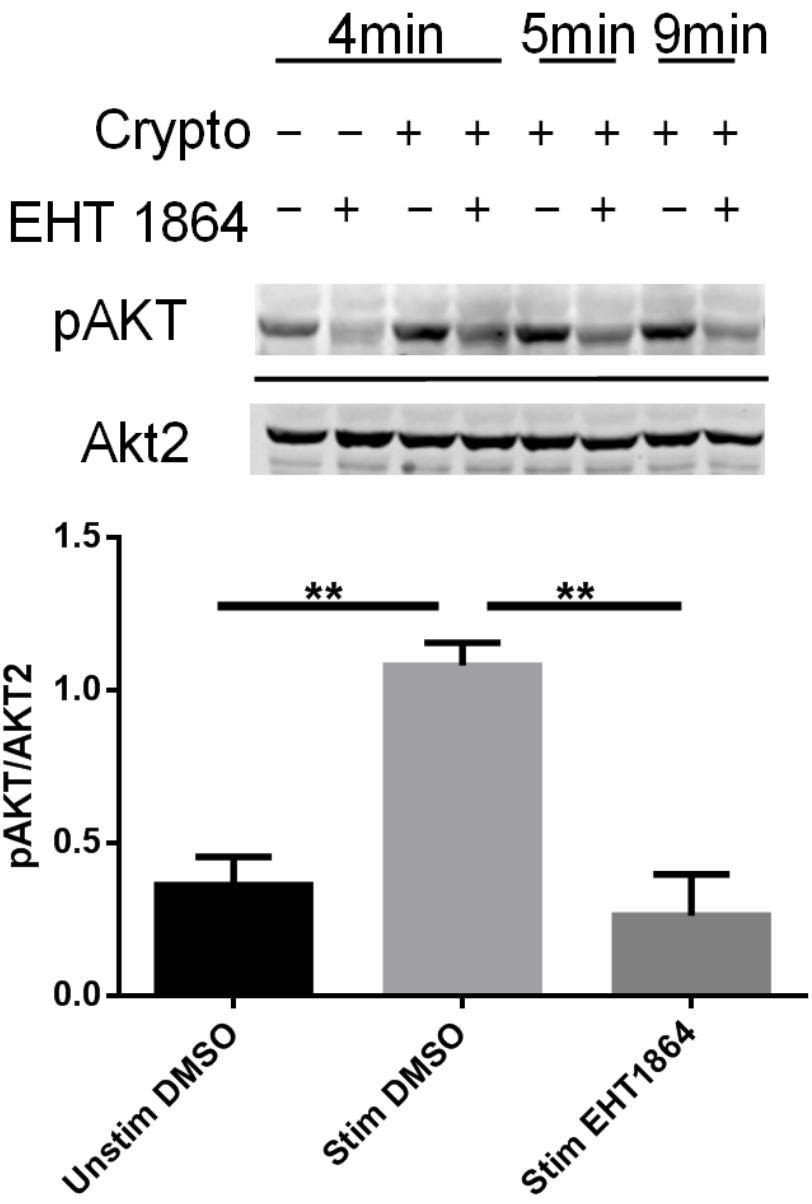


Figure 3-11: Rac1 small molecule inhibitor inhibits cryptococcal activation of PI3K. YT cells were pre-incubated with EHT 1864 or DMSO (-). YT cells were stimulated with *C. neoformans* for the indicated times. Top: Levels of pAkt and Akt2 were determined by immunoblots. Bottom: Densitometry of Akt phosphorylation at 4 minutes of stimulation. Bars are means of 3 experiments ± SEM. ** $p<0.01$

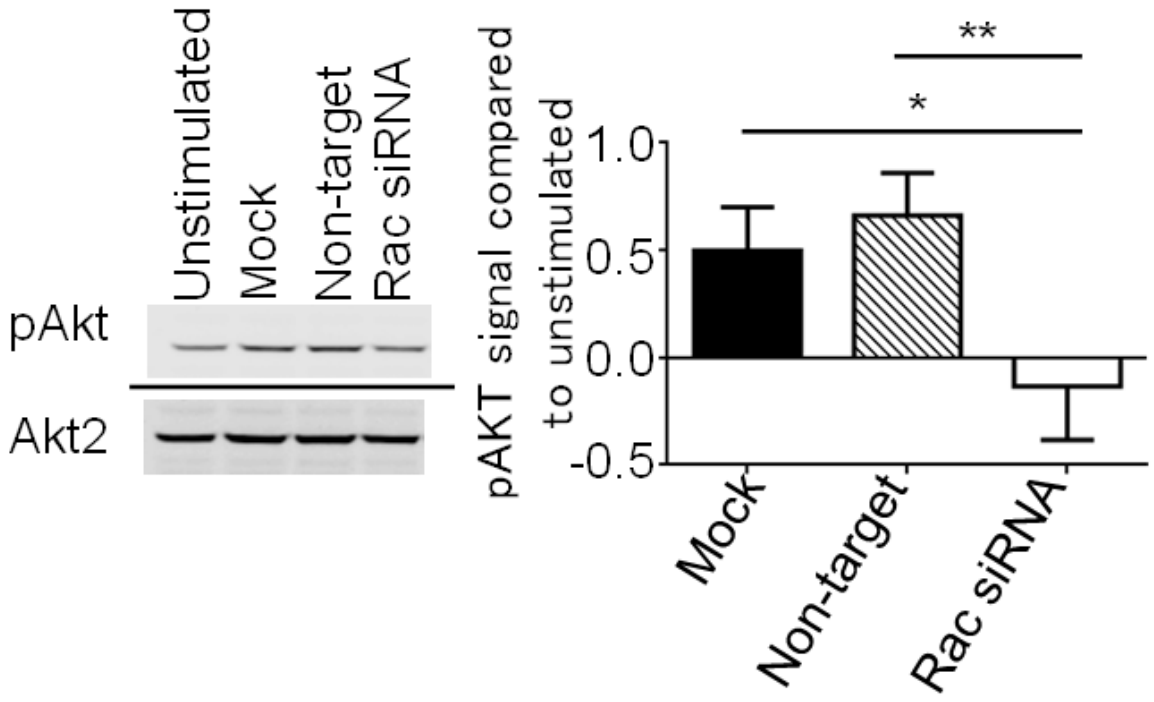


Figure 3-12: Rac1 siRNA knockdown inhibits cryptococcal activation of PI3K. YT cells were transfected with Rac1 siRNA. Left: Transfected YT cells were stimulated with *Cryptococcus* and the levels of pAkt were determined by immunoblots. Right: Densitometry is the mean of 3 experiments ± SEM. * $p<0.05$. ** $p<0.01$

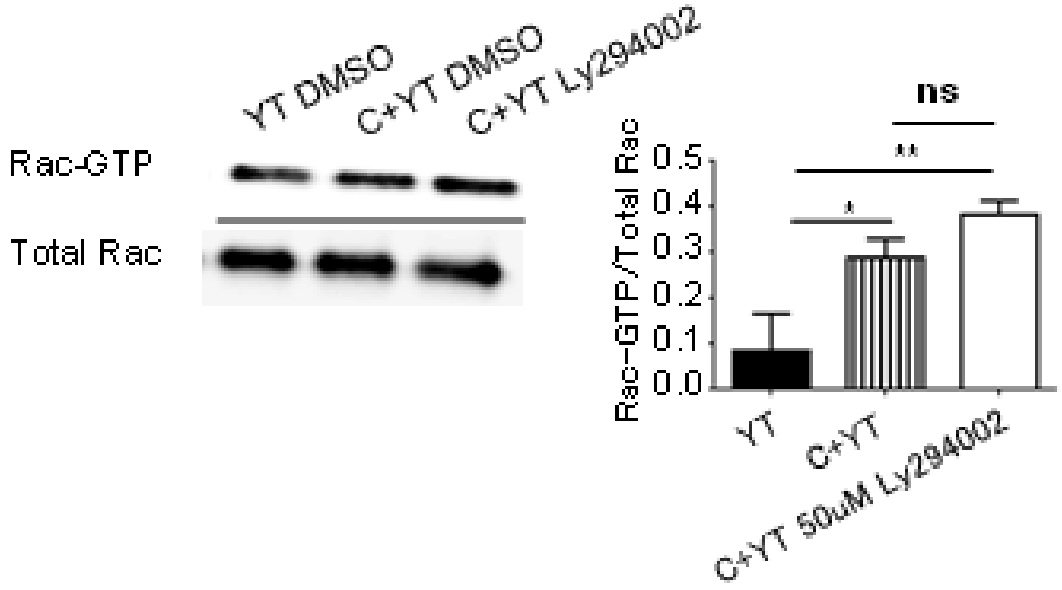


Figure 3-13: PI3K activity is not required to activate Rac1. YT cells were pre-incubated with 50uM Ly294002 or DMSO control. Left: Levels of active Rac bound to GTP were measured by Rac-GTP pull down assay and Rac1 immunoblotting. Aliquots of whole cell lysate were immunoblotted to determine the total amount of Rac1. Right: Densitometry is mean of 3 experiments ± SEM. YT: Unstimulated YT cells alone. C+YT: YT cells stimulated with *Cryptococcus*. *p<0.05. **p<0.01. ns: no significant difference.

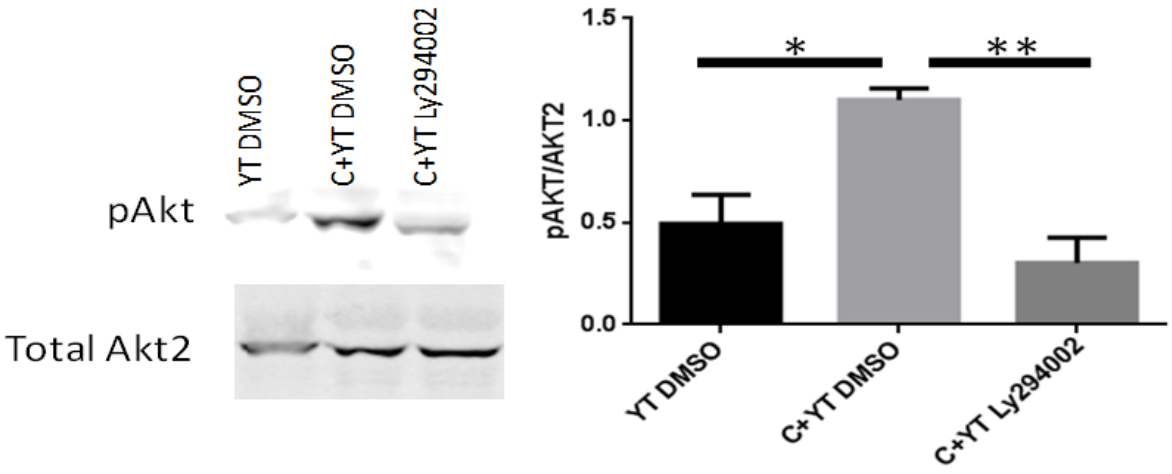


Figure 3-14: PI3K inhibitor inhibits PI3K activity. Left: Levels of phosphorylated Akt and total Akt2 were determined by immunoblots. Right: Densitometry is mean of 3 experiments ± SEM. YT: Unstimulated YT cells alone. C+YT: YT cells stimulated with *Cryptococcus*

Rac and SFK activate anti-cryptococcal activity through independent signaling pathways

Previous studies have established that SFK are required for the activation of the PI3K→ Erk signaling cascades in NK cell mediated cryptococcal killing [375,376]. Since inhibition of Rac1 prevented PI3K activity, I hypothesized that Rac1 could be distal to SFK, but proximal to PI3K. Therefore, I examined the interactions between Rac1 and SFK. YT cells were pretreated with EHT1864 or dasatinib, and then stimulated with *C. neoformans*. I performed immunoblots for phosphorylated SFK and found that Rac1 inhibition had no effect on SFK activation (Figure 3-15), but remarkably, SFK were not required to activate Rac1 in response to cryptococcal stimulation (Figure 3-16). These results suggest that SFK and Rac1 are independent pathways. Based on prior data that SFK activate PI3K in cryptococcal killing [285], both SFK and Rac1 are necessary for PI3K dependent NK cell cytotoxicity against *Cryptococcus.*

Conjugate formation between NK and *Cryptococcus* is SFK dependent

I found that conjugate formation between NK cells and *Cryptococcus* was independent of Rac1, and that *Cryptococcus* stimulation independently activates SFK and Rac1 pathways. This raised the possibility that conjugate formation depended on SFK signaling rather than Rac1. Previously, our lab had found that dasatinib caused a minor reduction in conjugate formation between fixed YT cells and *Cryptococcus* that did not achieve statistical significance [376]. Using a flow cytometry based assay and live cells, I found that pre-incubating YT cells or primary NK cells with the SFK inhibitor, dasatinib, reduced the proportion of NK cells in conjugate with *Cryptococcus* compared

to control, while EHT 1864 did not (Figure 3-17). The effect of dasatinib was more noticeable than in the previous study. Another SFK inhibitor, PP2, also reduced conjugate formation between YT cells and *Cryptococcus* compared to control PP3 (Figure 3-17). These findings suggest that *Cryptococcus* initiates a SFK signaling pathway in NK cells that leads to enhanced conjugate formation.

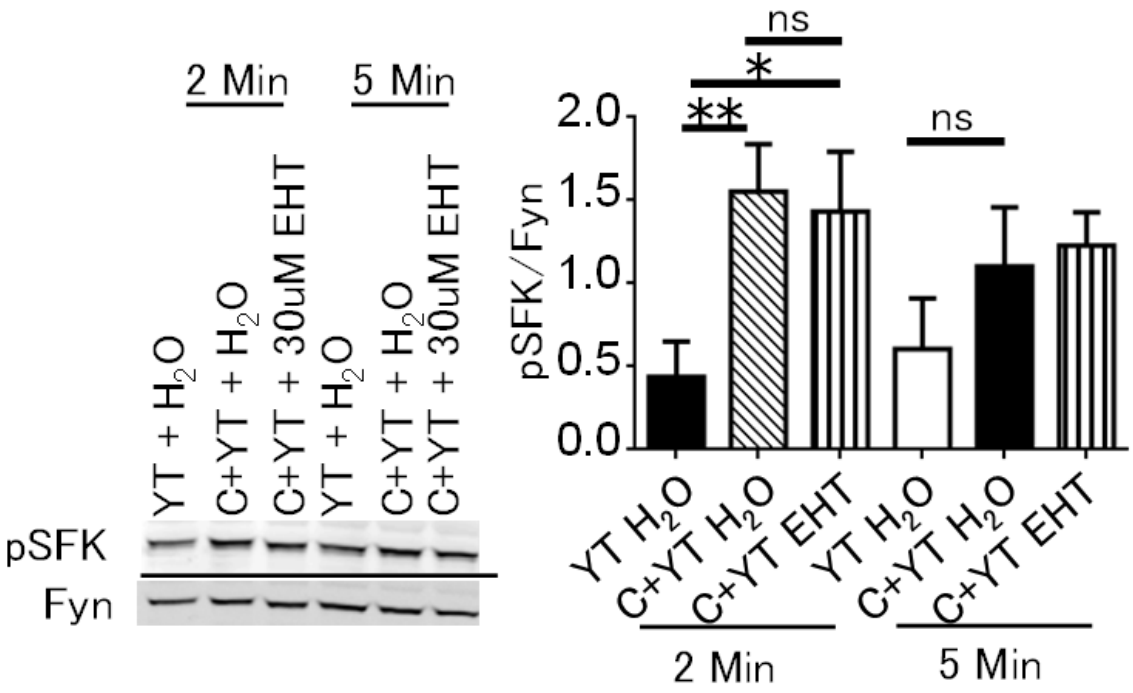


Figure 3-15: SFK activation is not dependent on Rac activity. YT cells were preincubated with EHT1864 and then stimulated with *Cryptococcus*. Left: Cells were lysed and immunoblots for pSFK and Fyn were performed. Right: Densitometry data bars are mean of 3 experiments ± SEM.*p<0.05, ** p<0.01. n.s. No statistically significant difference

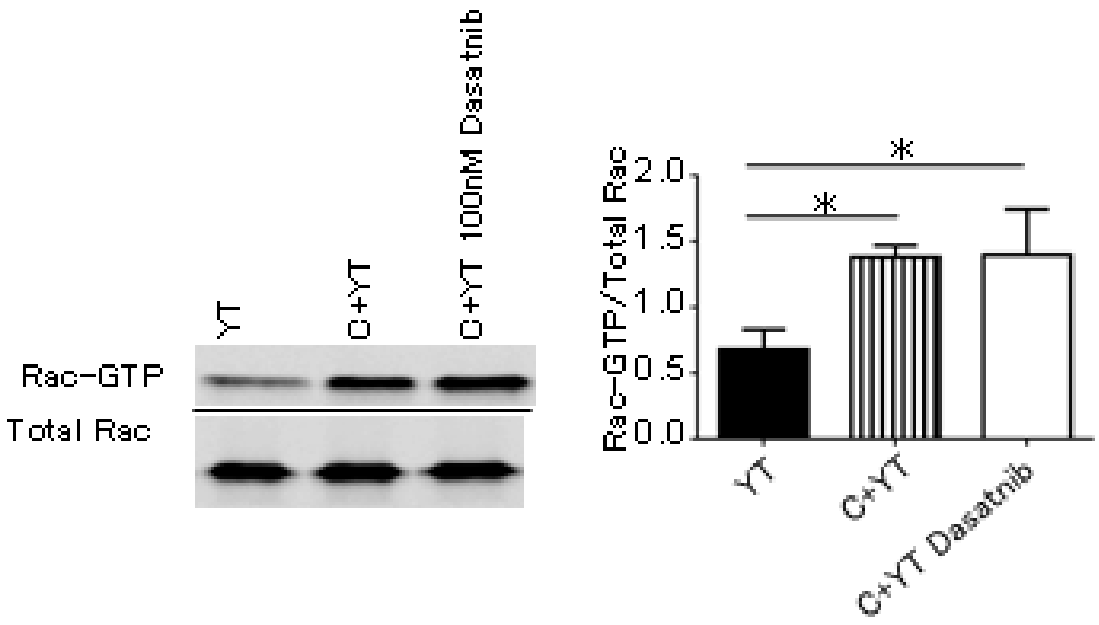


Figure 3-16: Rac activation is not dependent on SFK activity. YT cells were preincubated with dasatinib or control. Levels of Rac-GTP were determined by Rac-GTP pull down assay and Rac1 immunoblotting. Levels of total Rac were measured from whole cell lysate. Data are representative of 3 experiments. C+YT: YT cells stimulated with *Cryptococcus.* *p<0.05

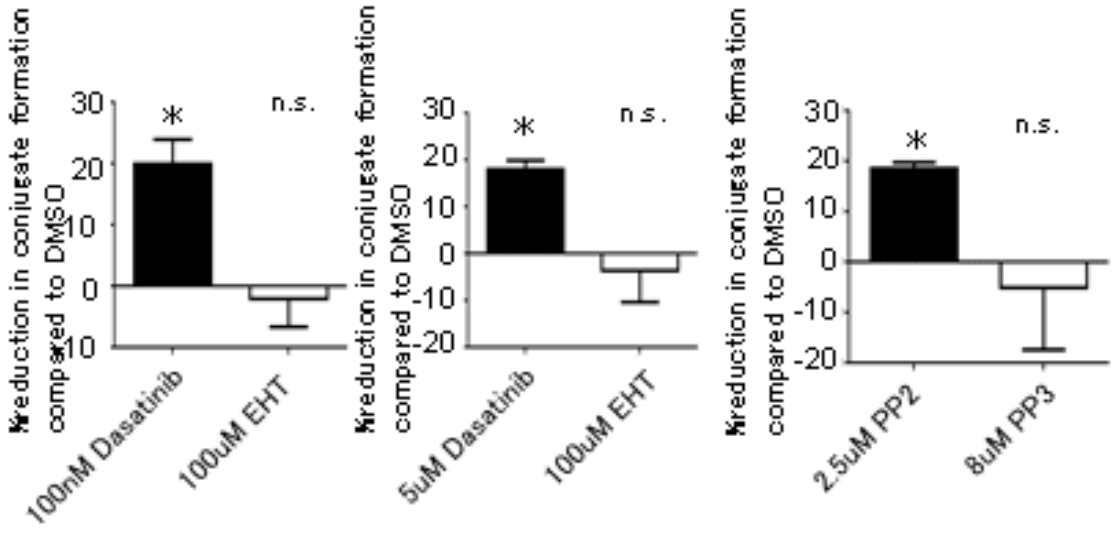


Figure 3-17: SFK, but not Rac1, is involved in NK-*Cryptococcus* conjugate formation. YT cells (Left) or primary NK cells (Center) were preincubated with dasatinib, EHT1864, or vehicle control. Right: YT cells were preincubated with PP2, PP3, or DMSO. YT cells and primary NK cells were then labelled with anti-CD11a (red) and *Cryptoococcus* was labeled with FITC. The percentage of cells forming conjugates was calculated by (number of green and red events)/(total number of green events). The percentage reduction in conjugate formation compared to DMSO was calculated by: (1-(% conjugates in treated)/(%conjugates in DMSO))*100%. Data are representative of 3 experiments (Left and Right) or 2 experiments (Center). *$p<0.05$, ** $p<0.01$. n.s. No statistically significant difference

3.4 Discussion

In this chapter I made 4 important observations exploring the non-canonical role of Rac1: i) *C. neoformans* stimulates NK cells and activates Rac1, ii) in contrast to tumor cytotoxicity Rac1 is upstream and required for the activation of the PI3K → Erk signaling pathway that leads to fungal cytotoxicity, iii) Rac1 and SFK independently and non-redundantly activate PI3K, iv) NK cell cryptococcal conjugate formation is dependent on SFK and occurs independent of Rac1.

Previous studies have shown that NK cell anti-cryptococcal killing and NK anti-tumor killing have many similarities. NK cells required PI3K → Erk signaling in both tumor and cryptococcal killing. NK microbial killing also depended on the NK activating receptor, NKp30 and the effector molecule, perforin [375,388], which are both used in tumor killing. I hypothesized that Rac1 may be involved in cryptococcal killing, since Rac1 was shown to be involved in actin polymerization and granule polarization in tumor killing. However, there were also fundamental differences between NK cell microbial killing compared to tumor killing. The binding interface between NK cells and fungal targets was different [335]. NK cells bound tumor targets with a tight interface in close apposition, while fungal targets were bound more distantly and appeared to use microvilli to penetrate the fungal target [393]. Additionally, while LFA-1 played a critical role in tumor cell killing, it did not play a role in killing of *Cryptococcus [394]*. In this chapter I showed that Rac1 was involved in cryptococcal killing; however, its role was distinct from tumor killing since it was proximal rather than distal to PI3K.

Having established that Rac1 was required for cryptococcal killing, I questioned how Rac1 affected NK cell function. Studies using other lymphocytes as well as NK

cells showed that Rac1 was important for conjugate formation [387,395,396]. Surprisingly, inhibiting Rac1 did not affect the ability of NK cells to form conjugates to *Cryptococcus* (Figure 3-7). As mentioned above, LFA-1, which has also been implicated in conjugate formation of NK cells, was not involved in cryptococcal killing [397,398]. Although conjugate formation can be achieved by other signaling pathways, the lack of Rac1 and LFA-1 involvement may suggest an unidentified actin remodeling process, which will be explored in a following chapter [397].

In addition to conjugate formation, I investigated the role of Rac1 in Erk activation, which is required for cryptococcal killing [375]. I found that inhibition of Rac1 prevented *Cryptococcus* from stimulating Erk phosphorylation, suggesting that Rac1 was required to activate Erk, as it is in tumor killing (Figure 3-9). Surprisingly, I also found that Rac1 was required for PI3K activity, but PI3K was not required for Rac1 activation (Figure 3-11,3-12,3-13), which is distinct from tumor killing. These findings demonstrated a non-canonical Rac1 signaling pathway. PI3K activation of Rac1 is well documented; however Rac1 activation of PI3K is less understood. Previous studies suggest models of Rac1 mediated activation of PI3K. Rac1 activation of PI3K have found that in mammalian epithelial cells (T47D), Rac1 activated PI3K leading to increased mobility and invasive properties [390]. In the chicken B cell line (DT40) B cell receptor stimulation initiated a Vav3 → Rac1 → PI3K pathway [399]. Also, active Rac1-GTP bound to PI3K directly in neutrophils and fibroblasts [400]. Rac1 and PI3K could also activate each other in a positive feedback loop, which allowed neutrophils to establish a leading edge [392]. Lastly, Rho family GTPases downstream of PI3K could regulate PI3K activation through a positive feedback loop [401]. My data added to this body of

literature by showing that Rac1 activation was proximal, required, but not sufficient for PI3K activation in cryptococcal killing. Additionally, I showed that in the Rac1 → PI3K pathway, knockdown of the Rac1 isoform prevented cryptococcal killing by NK cells. Although NK cells express both Rac1 and Rac2 isoforms [402], which share redundancy in T cells [403], loss of Rac1 was sufficient to prevent cytotoxicity. This suggested that Rac2 was not able to compensate for Rac1 in NK mediated cytotoxicity.

SFK have been shown to play a vital role in NK cell-mediated cryptococcal killing, by activating the PI3K → Erk signaling pathway [376]. Since both SFK and Rac1 were required for PI3K activation and SFK and Rac1 did not activate each other (Figure 3-15,3-16), these data indicated that SFK and Rac1 activated PI3K via independent signaling pathways. Interestingly, NKp30 relied on SFK-dependent CD3ζ signaling in tumor killing [202]. While there are no examples of natural cytotoxicity receptors, such as NKp30, activating both SFK and Rac-dependent pathways, I acknowledge that following TCR ligation and CD3ζ activation, ZAP70 activates Vav1 via linker of activated T cells (LAT) leading to activation of Rac1 [404]. Nevertheless, I believe my results are more consistent with a model whereby NKp30 activates SFK, while Rac1 is activated by another receptor, which I will explore in a following chapter.

The finding that SFK and Rac1 were activated independently also raised questions about the function of these pathways in NK cryptococcal killing. While both proteins have been found to activate cytotoxicity through Erk, I found that only SFK was responsible for conjugate formation between NK cells and *Cryptococcus* (Figure 3-17). This suggested that in addition to the Erk cytotoxicity pathway, SFK were required for the activation of a separate pathway that was responsible for conjugate formation. SFK

have also been shown to be required for conjugate formation between NK cells and tumor targets [405]. Additionally, in T cells, SFK have been shown to remodel the actin cytoskeleton and enhance conjugate formation between T cells and antigen-presenting cells (APC) via a PI3K → Erk independent pathway [406].Together, my data and previous findings pointed to the possibility that SFK regulated conjugate formation between NK cells and *Cryptococcus*, in a PI3K → Erk independent manner.

Since the cryptococcal stimulus induced Rac1 and SFK signaling pathways, which are independent of each other, my data uncovered a model of NK microbial cytotoxicity that was triggered by the simultaneous activation of multiple stimulatory pathways that worked together to activate PI3K. While I did not determine the mechanism by which Rac1 and SFK activated PI3K, the p85 subunit of PI3K is essential in NK cryptococcal killing [375], and Rac1 and SFK have been shown to directly bind to this subunit and activate PI3K [400,407]. Alternatively, Rac1 has also been shown to directly bind and activate the p110β subunit of PI3K [408]. Therefore, full activation of PI3K could require SFK to bind the p85 subunit and Rac to bind the p110β subunit. This model is similar to G-protein activation of the p110β subunit of PI3K, where full activation required both Gβγ and tyrosine kinase activity [408,409]. Rac and SFK may also activate PI3K in different cellular compartments. Since Rac1 can activate p110β subunit of PI3K directly, it might be able to activate PI3K with different regulatory subunits (p85, p55, or p50). On the other hand, SFK requires the p85 subunit of PI3K. The smaller (p55 and p50) regulatory subunits of PI3K localize to different compartments of the cell. For example, p55 binds to tubulin and localizes to the microtubule network [410]. The requirement for both SFK and Rac1 could be to allow

the cell to regulate where the activation of PI3K occurs.

The requirement for multiple stimulatory pathways is supported by previous studies on NK tumor killing. P815 redirected activation assays demonstrated that the NK receptors NKp46, 2B4, NKG2D, DNAM, and CD2 act synergistically to induce calcium flux, and degranulation [411]. Individually, each of these pathways was not enough to trigger calcium flux or degranulation, but co-crosslinking of multiple receptors activated a calcium influx pathway that resulted in degranulation. Each of these receptors utilized its own independent signaling pathways, which converged to promote NK cell function. This convergence of independent signaling pathways is reminiscent of my results. Individually, SFK and Rac1 were not capable of initiating the PI3K cytotoxicity pathway. However, the requirement for the activation of multiple signaling pathways could hinder NK cell-mediated microbial cytotoxicity, since a microbe that is able to evade even a single pathway could escape NK cell cytotoxicity. This raises the question why NK cells would require such stringent criteria in order to recognize and kill microbes. One possible explanation is that NK cells require multiple activation pathways to protect against targeting host cells if only one NK receptor was engaged non-specifically [412,413].

The involvement of Rac1 in NK cell mediated cryptococcal killing may provide clinical insights into the susceptibility to cryptococcosis in patients taking azathioprine [414,415]. In addition to being a purine analogue, azathioprine functions by blocking GTP binding to Rac1 and prevents Rac1 activation [416,417]. Patients treated with azathioprine have been reported to develop both pulmonary cryptococcosis and cryptococcal meningitis [414][415]. My data suggest that the increased susceptibility to

Cryptococcus could be caused in part by defective NK cell function, due to azathioprine-induced blockage of the Rac1 → PI3K → Erk cytotoxicity pathway.

Originally, I hypothesized that Rac1 was the intermediate molecule that allowed PI3K to activate Erk. Since I have shown that Rac1 is not activated by PI3K, it raised questions about how PI3K activates Erk in cryptococcal killing. In addition, although PI3K is activated by Rac1, this does not preclude other essential signaling pathways that might also be activated by Rac1, especially if these alternative pathways synergize with cytotoxic functions that are controlled by Erk activity. Although I have shown that Rac1 was not involved in conjugate formation between *Cryptococcus* and YT cells, Rac1 has been shown to be involved in actin and microtubule remodelling, which could make it important for granule trafficking that occurs in later stages of cytotoxicity. One possibility is that Rac GTPases are involved in the phosphorylation of stathmin, which is required for microtubule remodelling [418]. Additionally stathmin also causes microtubule-organizing center (MTOC) polarization in activated T cells [419], so there is the possibility that cryptococcal mediated MTOC polarization in NK cells is regulated by a Rac1→ stathmin pathway.

In summary, I have demonstrated a novel mechanism by which Rac1 activated PI3K in NK cells, and that this pathway was required for direct microbial cytotoxicity. This furthers our understanding of Rac1 as an upstream activator of PI3K, and defines a novel role for Rac1 in NK microbial cytotoxicity.

Chapter 4: β-1

integrins signaling in anti-cryptococcal killing

4.1 Contributions

Some purification of NK cells was done by Danuta Stack.

4.2 Introduction

NK mediated tumor killing depends on both activating receptors and integrins, that mediate different roles in cytotoxicity [420]. It has been shown that FcRγ, an ITAM containing adaptor protein, allows for fusion of cytotoxic granules with the plasma membrane, while integrin signaling allows for granule polarization towards the immune synapse [10]. An ITAM adaptor dependent anti-cryptococcal receptor, NKp30, has been identified to recognize *C. neoformans* and initiate killing [262]. However, whether an integrin is required for granule polarization against *Cryptococcus* is unknown.

The primary class of integrins involved in tumor killing are β-2 integrins such as LFA-1 or macrophage-1 antigen (Mac-1). Although LFA-1 and Mac-1 facilitate tumor killing by enhancing adherence [421] and perforin polarization towards the IS [422], they were shown not to be involved in cryptococcal killing [394]. The next most likely class of integrins to be involved in cryptococcal killing are β-1 integrins. β-1 integrins are

capable of forming heterodimers with the largest number of α chains. β-1 integrins also have the capacity to bind to fungal β-glucans [423]. Specifically, blocking β-1 integrins with extracellular matrix protein antagonists prevented neutrophil activation by poly-[1-6]-D-glucopyranosyl-[1-3]-D-glucopyranose (PGG) glucan [424]. Since *Cryptococcus* also expresses similar β-glucans, it suggests that β-1 integrins could be an anti-cryptococcal adhesion molecule.

Our lab has shown that upon cryptococcal stimulation, NK cells activates both SFK [376] and Rac1 [425] signaling and that both signals are required to activate a cytotoxic PI3K → Erk pathway. The receptors responsible for activating SFK and Rac1 are unknown. However, NKp30 was found to recognize *Cryptococcus*, and activate the PI3K→ Erk pathway [388]. However, it is unclear if NKp30 is activating PI3K through SFK or Rac.

In this chapter, I investigate the role of β-1 integrins in cryptococcal killing, and how NKp30 activates the PI3K → Erk cascade in response to *Cryptococcus*.

4.3 Results

Cryptococcal stimulation activates NKp30 and signals through Src family kinases

Previous studies have found that SFK and Rac are upstream of the PI3K → Erk anti-cryptococcal cascade in NK cells, and that NKp30 is also required for cryptococcal killing through the same PI3K → Erk signaling pathway. However, it is unknown how NKp30 activates PI3K. We investigated whether NKp30 is the receptor responsible for the activation of SFK or Rac. YT cells were co-cultured with *Cryptococcus* in the presence of an inhibitory anti-NKp30 antibody (clone 1C01) and SFK signaling was

tested after cryptococcal stimulation (Figure 4-1). Immunoblotting showed YT cells treated with 1C01 had impaired SFK activation in response to *Cryptococcus* - suggesting that NKp30 recognizes *Cryptococcus* and activates the SFK pathway (Figure 4-1). Other studies have also found that NKp30 signals through CD3ζ, which depends on SFK [202]. Unlike SFK, cryptococcal induced Rac activation was not inhibited by the addition of inhibitory anti-NKp30 (Figure 4-2), suggesting that NKp30 does not stimulate the Rac mediated pathway.

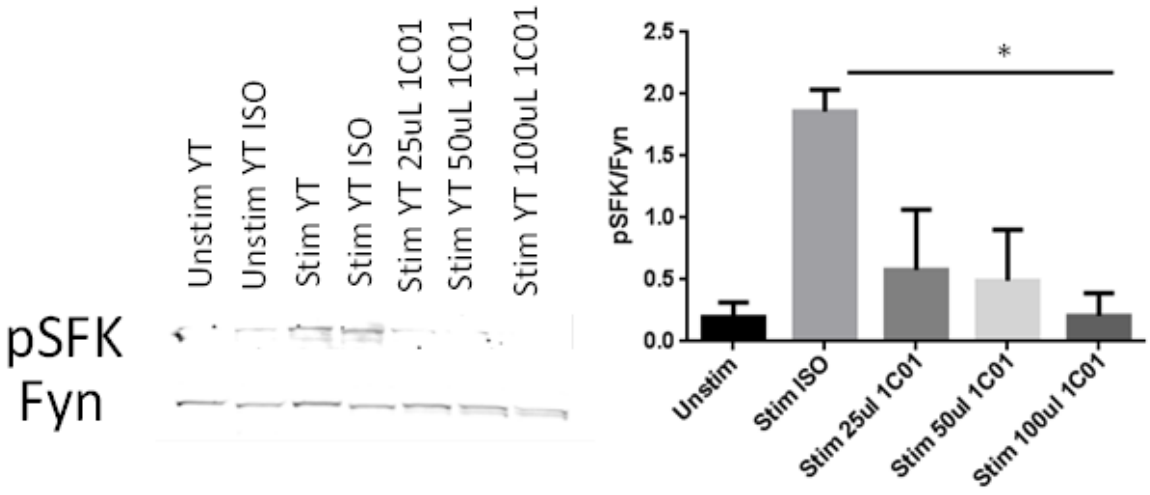


Figure 4-1:*Cryptococcus* activates SFK through NKp30. Left: YT cells were pre-incubated with various concentrations of anti-NKp30 for 30 minutes in a 37C CO_2 incubator. YT cells were then stimulated with *Cryptococcus* and lysed. Lysates were separated by SDS-PAGE and immunoblotted for activated SFK and total Fyn, as the loading control. Right: Densitometry of 3 independent immunoblots performed using the protocol in left panel. Bars are the mean ± SEM. *p <0.05

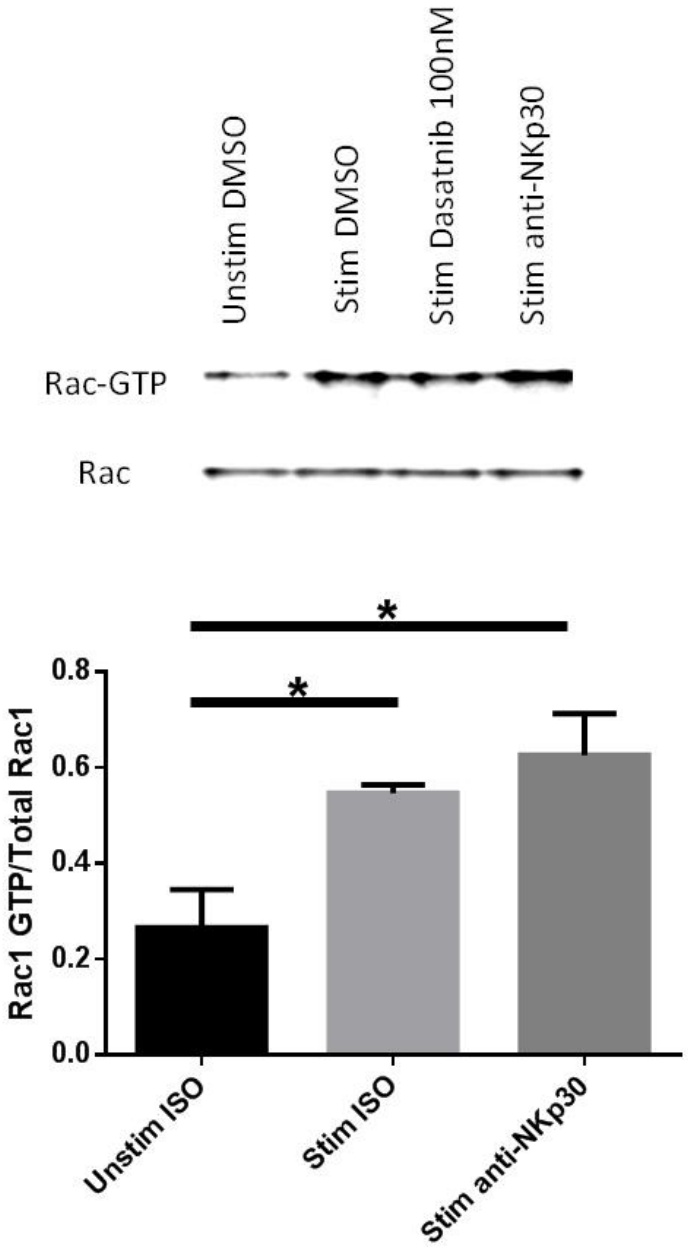


Figure 4-2: NKp30 is not necessary for Rac activation in cryptococcal killing. YT cells were treated with anti-NKp30 inhibitory antibody or a SFK inhibitor and then stimulated with *Cryptococcus.* YT cells were then lysed active Rac-GTP was extracted using a PBD pull down kit. Top: Levels of active Rac was measured by immunoblot. Bottom: Densitometry data bars are mean of 3 experiments ± SEM. *p <0.05

Integrin-linked kinase is required for Rac1 dependent NK anti-cryptococcal response

Integrins play major role in NK tumor cytotoxicity. Since integrins are also capable of activating Rac1, I examined if integrins are involved in Rac1 activation during cryptococcal killing [426,427]. First, I tested if integrin signaling occurred in response to cryptococcal stimulation. β-1 and β-2 integrins are known to activate ILK [428]. I stimulated YT cells with cryptococcus and investigated the activation of ILK by immunoblotting. Immunoblotting for active ILK found that stimulation with *C. neoformans* induced activation of ILK in YT cells (Figure 4-3). The small molecule inhibitor, CPD 22 was used to inhibit ILK activity. CPD 22 inhibits ILK activation and the phosphorylation of ILK targets [429]. CPD 22 is a specific inhibitor of ILK since the phosphorylation of the downstream targets was restored with the introduction of constitutively active ILK, and when tested at high concentrations against a panel of 20 kinases, CPD 22 did not significantly impact the activity of 19 of those kinases [429]. In order to test the involvement of ILK, YT cells treated with CPD-22 and then stimulated with *Cryptococcus* showed reduced Rac1 activation compared to control (Figure 4-4). This suggests that ILK activity is required for activation of the Rac pathway. Since Rac is involved in cryptococcal killing [263] and ILK activates Rac, we would expect ILK inhibition to impair cryptococcal killing. Indeed, I found that YT cells treated with CPD-22 showed reduced anti-cryptococcal activity (Figure 4-5). Taken together these findings suggest that integrins and their signaling pathways play a crucial role in NK mediated anti-cryptococcal killing.

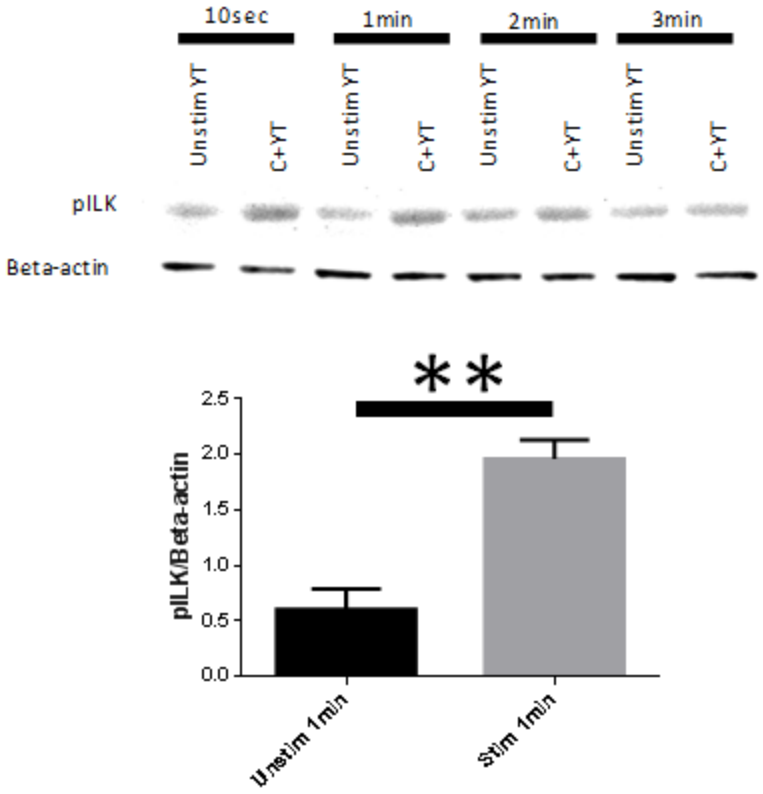


Figure 4-3: ILK is activated by cryptococcal stimulation. Top: YT cells were stimulated with *Cryptococcus* for varying time points and then lysed. Lysates were immunoblotted for p-ILK and β-actin. Bottom: Densitometry showing the mean of 3 independent experiments. Error bars represent SEM. ** $p<0.01$

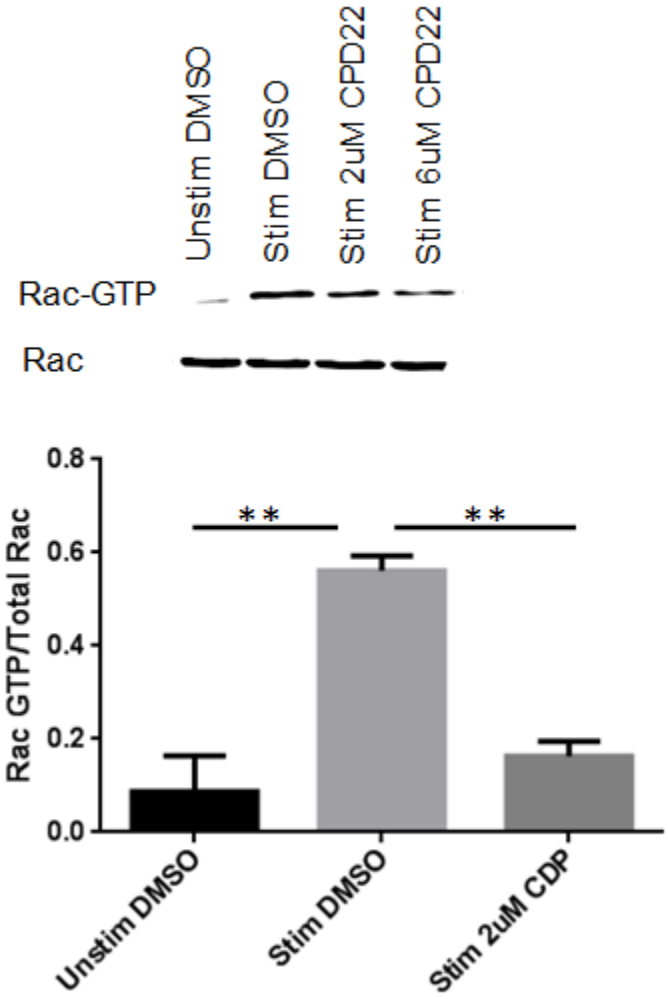


Figure 4-4: ILK is necessary for Rac activation after cryptococcal stimulation. Top: YT cells were pre-incubated with 2uM CPD 22 for 1 hour. YT cells were then lysed and levels of active Rac1 were measured by PBD pull down kit and immunoblotting. Bottom: Densitometry data of immunoblots testing Rac activation is presence of CPD 22. Bars are mean of 3 independent experiments ± SEM. ** $p<0.01$.

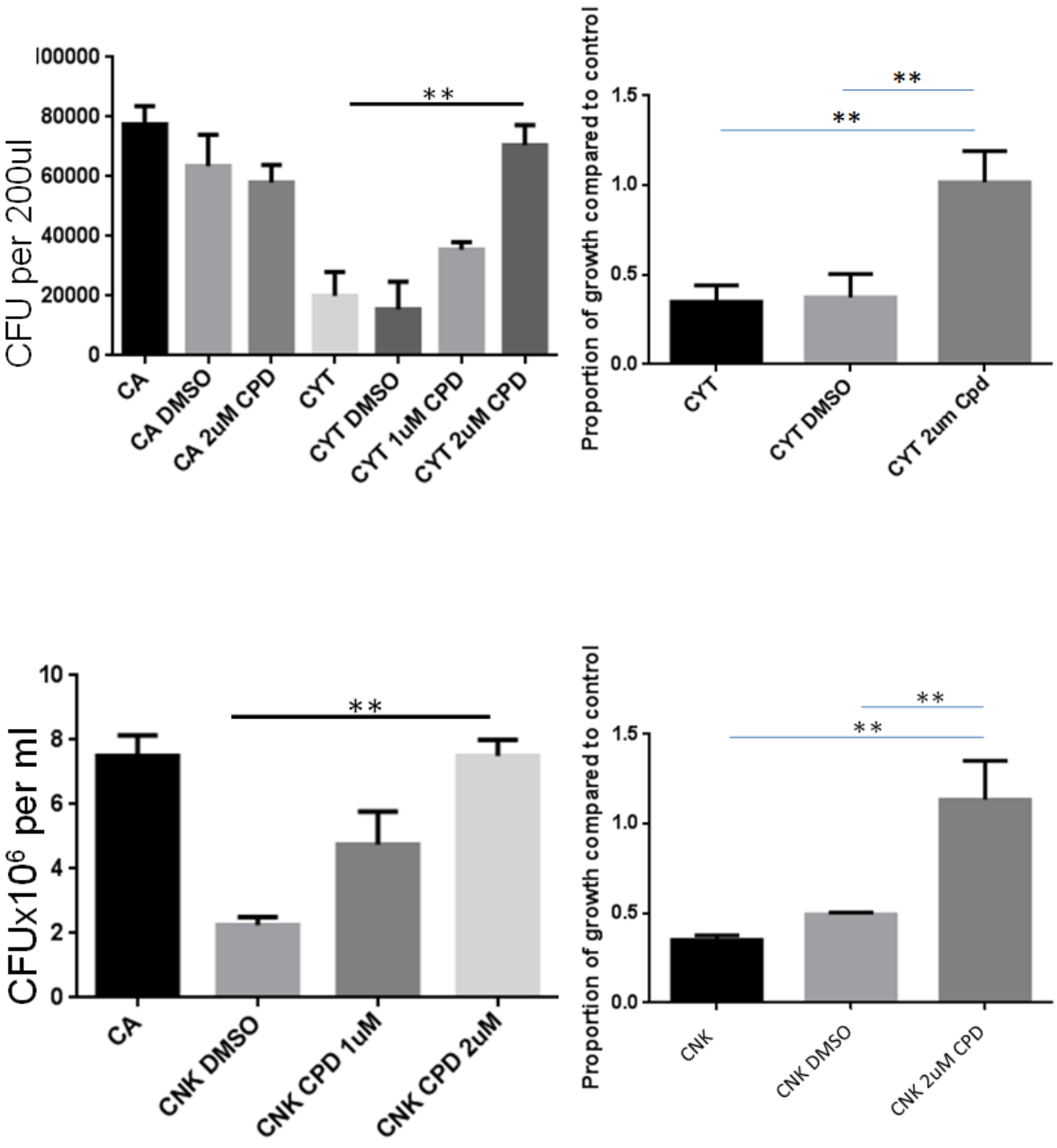


Figure 4-5: ILK activity is required for NK mediated cryptococcal killing. Top Left: YT cells were co-cultured with *Cryptococcus* in the presence of CPD 22 or control. Bars are means of quadruplicate wells in a single experiment ± SEM. Top Right: Inter-experimental statistics from 3 experiments. Bottom Left: Primary NK cells were co-culture with *Cryptococcus* in the presence of CPD 22 or DMSO. Bars are means of quadruplicate wells in a single experiment ± SEM. Data is representative of 3 independent experiments. Bottom Right: Inter-experimental statistics from 3 experiments. ** $p<0.01$.

β-1 integrins, but not β-2 integrins are required for NK mediated cryptococcal killing

Although multiple integrin classes can activate ILK signaling, I decided to investigate β-1 and β-2 integrins, since β-2 integrins are the primary class responsible for tumor killing and β-1 integrins form the largest number of α-β heterodimers. Both β-1 and β-2 integrins are expressed on NK cells and modulate NK cytotoxicity [430]. In order to determine the role of these integrins in NK anti-cryptococcal killing, I reduced their protein expression by siRNA knockdown (Figure 4-6). I found that YT cells with diminished expression of the β-1 integrin protein chain (CD29) had reduced anti-cryptococcal activity, while YT cells with diminished β-2 integrin chain (CD18) protein expression experienced no change in anti-cryptococcal activity (Figure 4-7, 4-8). The lack of β-2 integrin involvement agrees with previous literature that showed LFA-1 (CD11a/CD18) was not required for cryptococcal killing [394]. I further explored β-1 integrins by performing an anti-cryptococcal cytotoxicity assay in the presence of a blocking β-1 integrin antibody. I found that blocking β-1 integrins also reduced the anti-cryptococcal activity of YT cells and primary NK cells compared to isotype control (Figure 4-9). These findings suggest β-1 integrins are necessary for NK cell mediated anti-cryptococcal killing, but β-2 integrins are not. It also seems that less inhibitory antibody is required to inhibit YT cells than primary NK cells. However, this finding is likely due to the lower E:T ratio used in the YT cell killing assay compared to the primary NK cell killing assay.

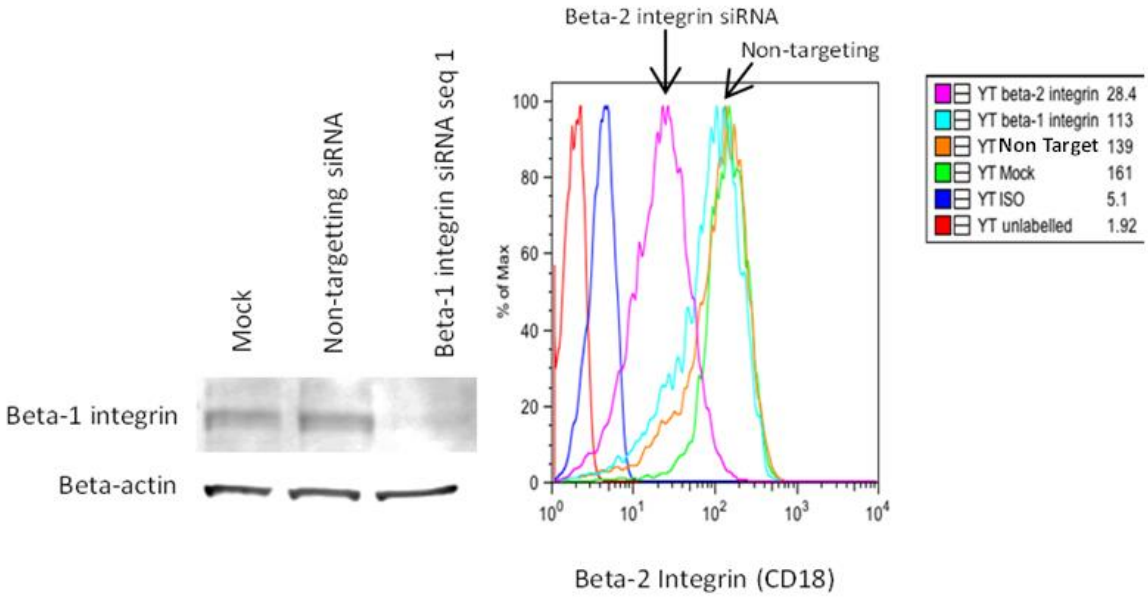


Figure 4-6: β-1 and β-2 integrin siRNA knockdown reduced the expression of those integrins in NK cells. YT cells were transfected with siRNA to β-1 integrin, β-2 integrin, or non-targeting or mock transfected. Left: Transfected YT cells were rested for 24 hours in a 37C CO_2 incubator. After 24 hours YT cells were lysed. Lysates were separated by SDS-PAGE and immunoblotted for β-1 integrin. Immunoblotting was chosen because the β-1 integrin antibody was not recommended for flow cytometry. Right: Transfected YT cells were labelled with anti-β-2 integrin antibody conjugated to PE-cy5. Surface expression of β-2 integrin was assessed by flow cytometry.

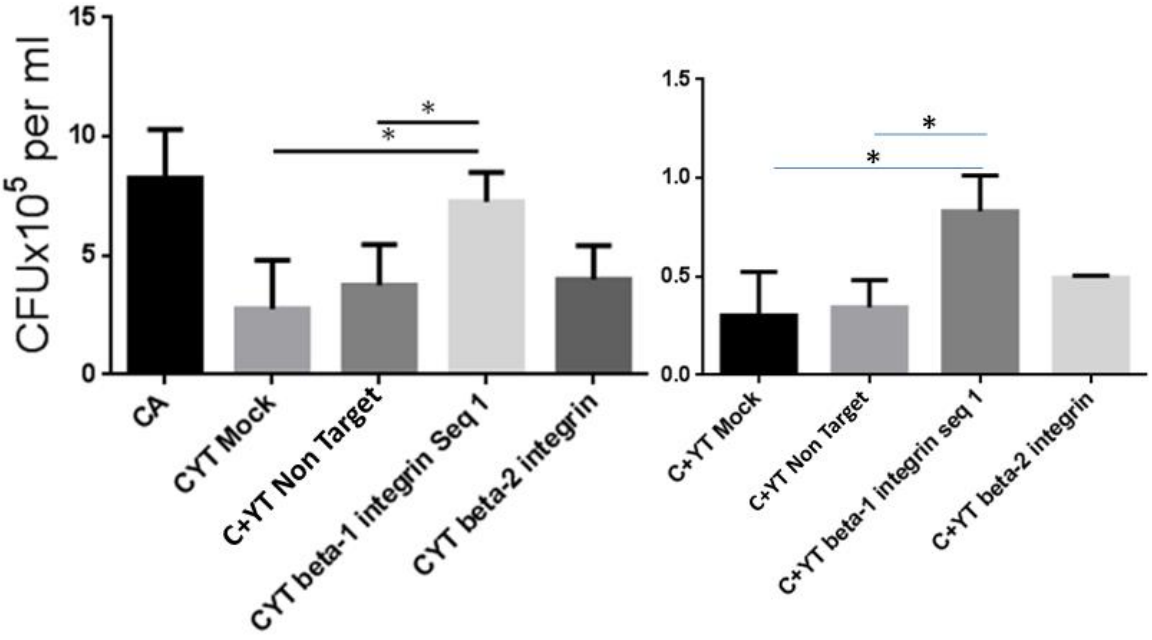


Figure 4-7: β-1, but not β-2 integrins are required for cryptococcal killing. Left: Transfected YT cells were co-incubated with *Cryptococcus* for 48 hours and the levels of cryptococcal growth was assessed by plating the *Cryptococcus* on Sabouraud dextrose agar plates and counting CFU.* $p<0.05$. Bars are means of quadruplicate wells in a single experiment ± SEM. Right: inter-experimental statistics from 3 experiments. Data is representative of 3 independent experiments.

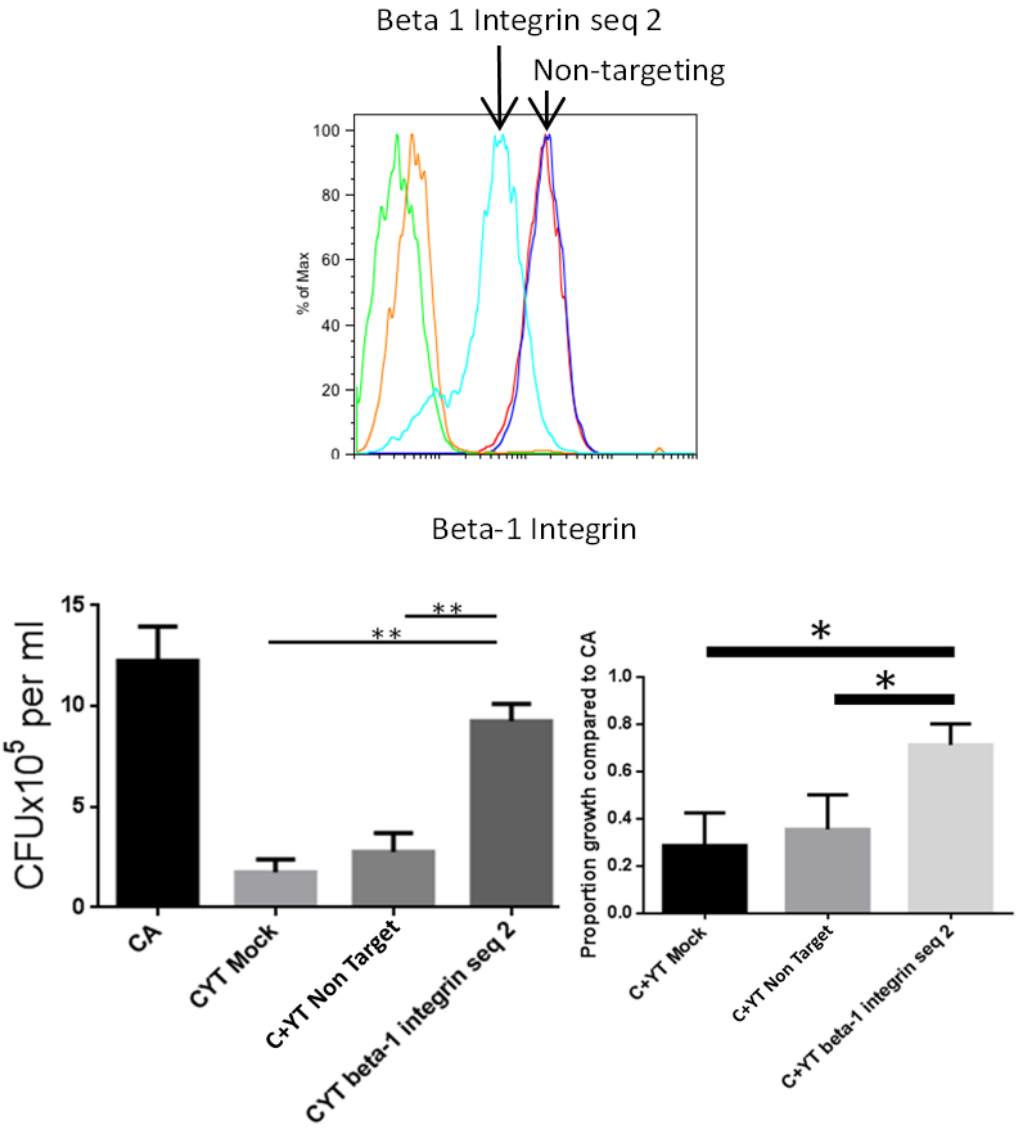


Figure 4-8: siRNA knockdown of β-1 integrins inhibits NK cell killing of *Cryptococcus*. Top: YT cells were transfected with a different second sequence of β-1 integrin siRNA. YT cells were then rested for 24 hours as described above. After 24 hours transfected YT cells were labelled with anti-β-1 integrin antibody conjugated to PE-cy5 that is appropriate for flow-cytometry. Surface expression of β-1 integrin was assessed by flow cytometry. Bottom Left: Rested transfected YT cells, with the second sequence against β-1 integrin, were co-incubated with *Cryptococcus* for 48 hours and anti-cryptococcal activity was assessed by plating the *Cryptococcus* on Sabouraud dextrose agar plates and counting CFU. Bars are means of quadruplicate wells in a single experiment ± SEM. Bottom Right: inter-experimental statistics from 3 experiments. ** $p<0.01$. Data is representative of 3 independent experiments

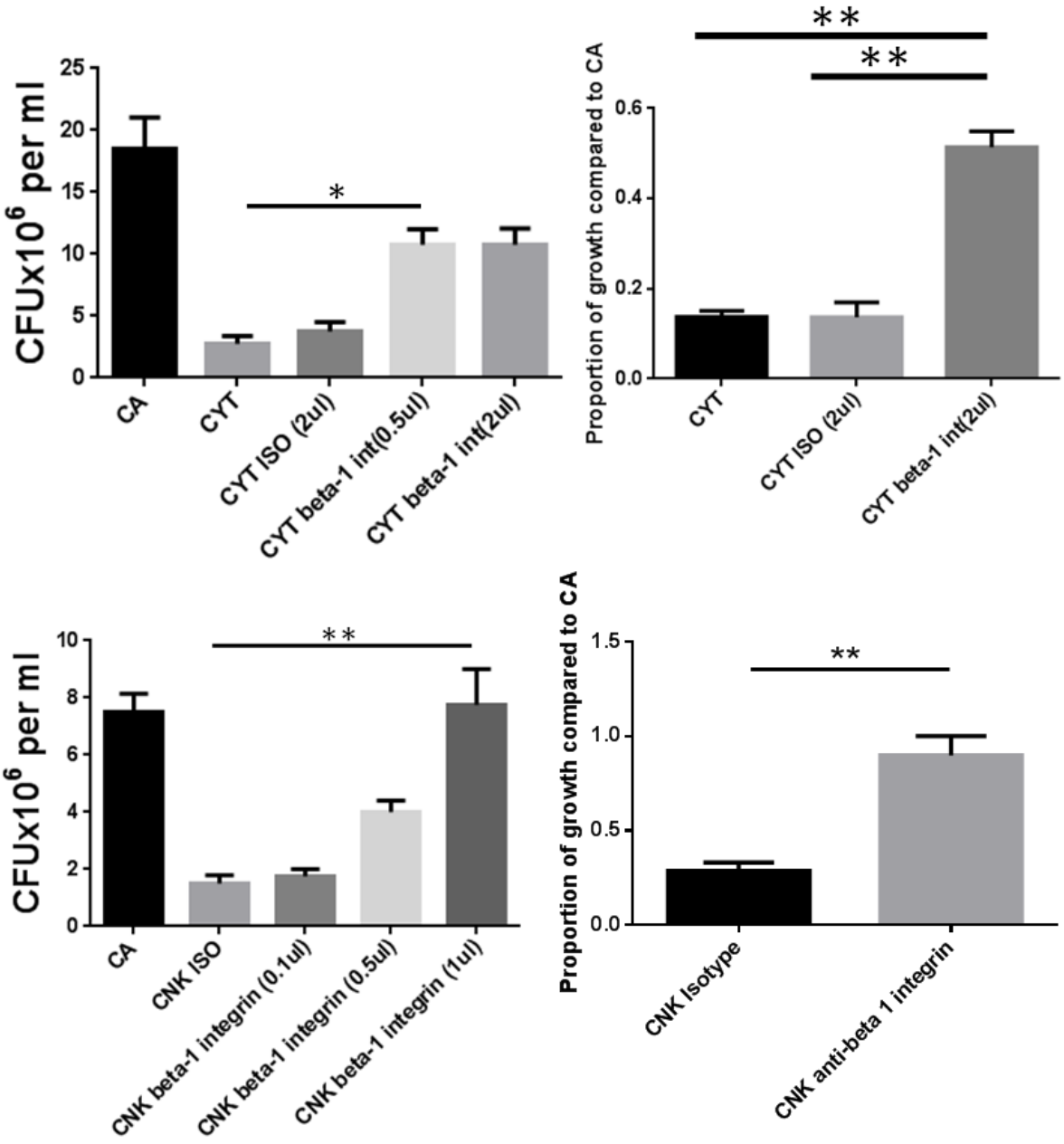


Figure 4-9: Blocking antibodies against β-1 integrins prevented NK cell killing of *Cryptococcus*. Top Left: YT cells were co-cultured with *Cryptococcus* in the presence of an inhibitory anti-β-1 integrin (CD29) antibody or isotype control for 48 hours. *Cryptococcus* was then plated on Sabouraud dextrose agar plates and CFU were counted. Bottom Left: Primary NK cells were co-cultured with *Cryptococcus* in the presence of an inhibitory anti-β-1 integrin antibody or isotype control. Top Right and Bottom Right: inter-experimental statistics from 3 experiments. Bars are means of quadruplicate wells in a single experiment ± SEM. Data is representative of 3 independent experiments. * $p<0.05$ ** $p<0.01$.

β-1 integrins are required to activate ILK in response to cryptococcal stimulation

The involvement of both ILK and β-1 integrins in cryptococcal killing suggested that β-1 integrins activate ILK. I examined if inhibition of β-1 integrin affected ILK activation. Using siRNA knockdown of β-1 integrin, I found that reduction in β-1 expression causes a reduction in ILK activation in response to cryptococcal stimulation (Figure 4-10). This suggests that *Cryptococcus* activates β-1 integrins to initiate an ILK → Rac1 signaling pathway. This finding is consistent with previous studies that also showed β-1 integrin signals through ILK [428].

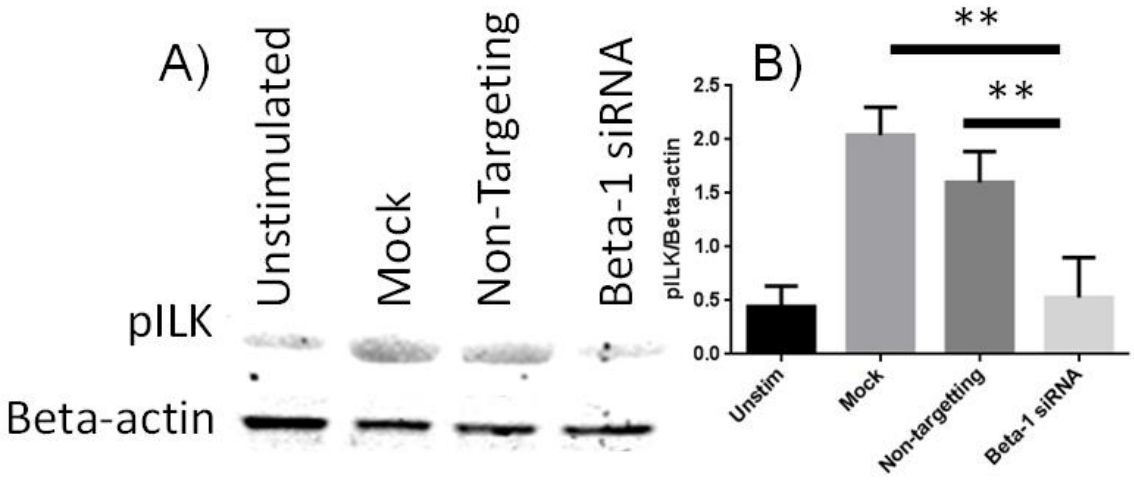


Figure 4-10: β-1 integrin is required for activation of ILK. YT cells were transfected with non-targeting or β-1 integrin siRNA, or mock transfected. YT cells were rested for 24 hours in a 37°C CO_2 incubator. YT cells were then stimulated with *Cryptococcus* for 5 minutes and then lysed. Left: Lysates were separated by SDS-PAGE and immunoblotted for pILK and β-actin. Right: densitometry is mean of 3 independent experiments ± SEM. ** $p<0.01$

Lipid rafts, but not β-1 integrins, are required for conjugate formation

In addition to their role in signaling, integrin are involved in adhesion between NK cell and tumor targets; specifically β-2 integrins [296]. Since β-2 integrins are not involved in cryptococcal killing, there is a possibility that β-1 integrins could be compensating for the lack of β-2 integrins and enhance conjugate formation between NK and *Cryptococcus*. Both β-1 and β-2 integrins are dependent on lipid rafts to mediate adhesion [431]. I found that disruption of lipid raft with MBCD reduced NK cell-*Cryptococcus* conjugate formation (Figure 4-11). Therefore, I proceeded to investigate if knockdown of the β subunit of β-1 integrins inhibited conjugate formation. I reduced the surface expression of β-1 integrin on YT cells by siRNA knockdown (Figure 4-8), and found that YT cells with reduced β-1 integrin expression did not have reduced conjugate formation compared to non-targeting siRNA or mock transfected (Figure 4-11). This suggests that β-1 integrins are not a major contributor of adherence to *Cryptococcus*. Therefore the reduction in NK cytotoxicity caused by β-1 integrin inhibition is likely the result of impaired signaling rather than reduced adherence.

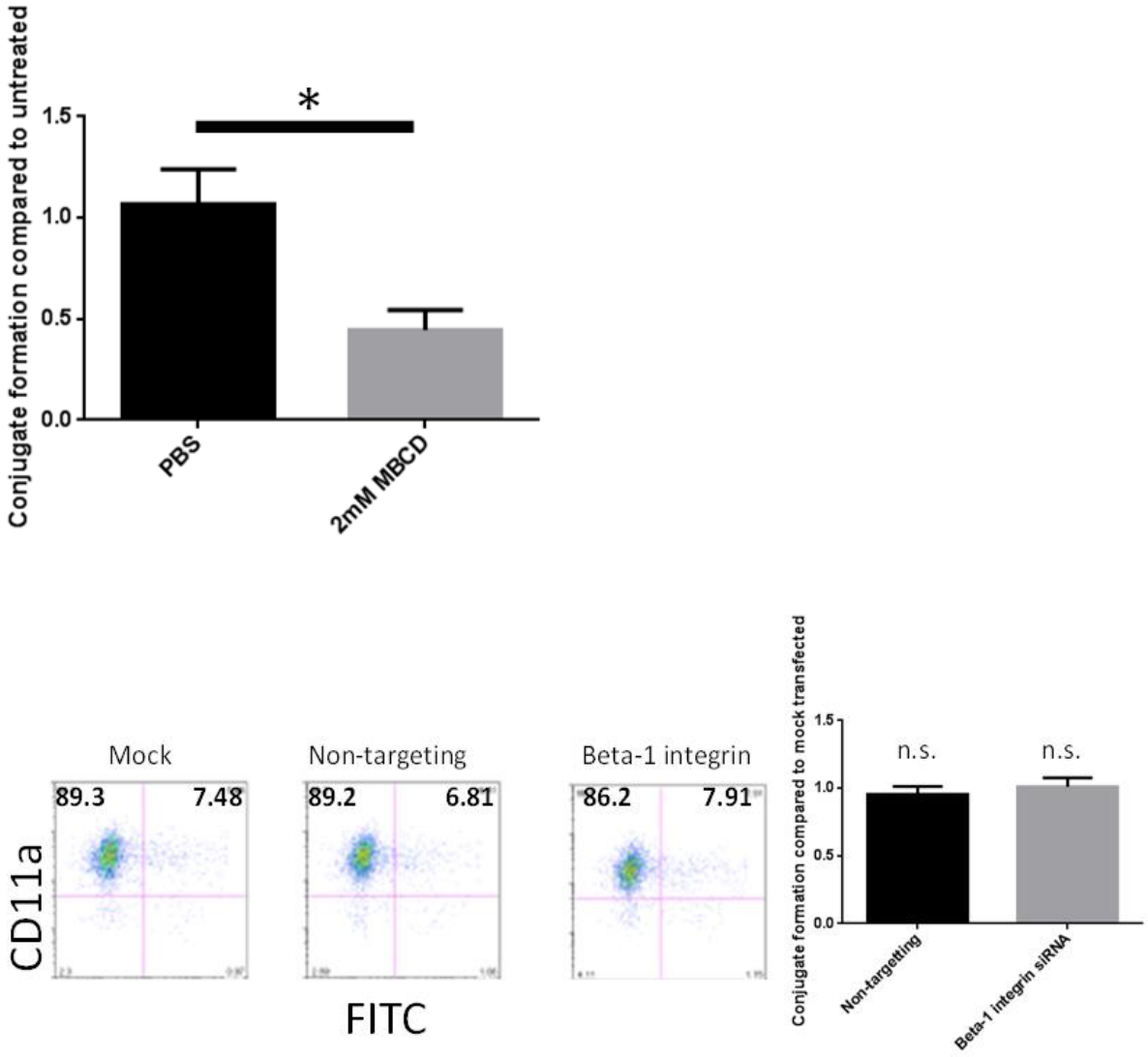


Figure 4-11: Knockdown of β-1 integrins does not affect NK-cryptococcal conjugate formation. YT cells and *Cryptococcus* were labelled with anti-CD11a and fitc respectively. YT cells and *Cryptococcus* were then co-cultured for 25 minutes at room temperature. Top: YT cells were preincubated with MBCD and then co-cultured with *Cryptococcus*. Conjugates were analyzed by flow cytometry. Bars are mean conjugate formation of 3 independent experiments ± SEM. Left: Cells were analyzed by flow cytometry. Conjugates were detected as double positive events. Right: Mean conjugate formation of 3 independent experiments ± SEM. n.s. No significant difference, * $p<0.05$

4.4 Discussion

In this chapter I showed that: i) the anti-cryptococcal receptor, NKp30, activates the SFK pathway, but not the Rac signaling pathway, ii) β-1 integrins are involved in NK mediated cryptococcal killing, iii) β-1 integrins induce an ILK → Rac signaling pathway that is required for anti-cryptococcal activity, iv) β-1 integrins are not required for conjugate formation between NK cells and *Cryptococcus*. Together these results describe a novel role of β-1 integrins in activating anti-cryptococcal cytotoxicity. Anti-cryptococcal killing has been shown to require both SFK and Rac signaling pathways [376,425]. I found that NKp30 is responsible for the activation of SFK (Figure 4-1) and that β-1 integrins activate the Rac1 signaling pathway through ILK (Figure 4-3 to 4-10). This suggests that cryptococcal killing is similar to tumor killing in that both NK activating receptors and integrins are required and that NKp30 activation of SFK is essential. However, the family of integrins is different from tumor killing. In tumor killing, the β-2 integrins - specifically LFA-1 - serve as adhesion molecules and signaling proteins that lead to granule polarization [10]. The impact of LFA-1 on granule polarization is independent of activation receptor signaling - such as from FcγR or NKp30. The role of β-1 integrins in tumor cytotoxicity is less clear. Studies have found that cross-linking β-1 integrins in NK cells can upregulate production of IFNγ [317], and IL-8 [318]. Cross-linking β-1 integrins have also been shown to enhance ADCC [315]. In this chapter I have demonstrated that direct activation of β-1 integrins by cryptococcal targets leads to enhanced cytotoxicity due to activation of the Rac signaling pathway. Since SFK are activated by NKp30, my data suggests that NK activating receptors and integrin signaling converges on a central cytotoxic cascade. This is different from the

tumor model where activation receptors and integrins controls independent components of cytotoxicity. Additionally, during cryptococcal killing β-1 integrins are not required for adhesion since loss of β-1 integrins did not affect conjugate formation or SFK activation (Figure 4-1 and 4-11). These data demonstrate a novel role of β-1 integrins in anti-fungal NK cytotoxicity.

The lack of β-1 (Figure 4-11) and β-2 integrins involvement in conjugate formation with *C. neoformans* [394] could explain why the conjugate between *Cryptococcus* and NK cells is weaker and its formation is delayed compared to tumor killing [393]. This lack of an integrin adhesion molecule could hinder cytotoxic signaling since it would be easier for the conjugate to be prematurely disrupted.

Our research has shown that cryptococcal stimulation activates β-1 integrin signaling; however the ligand responsible is unclear. β-1 integrin ligands are mainly matrix proteins like fibronectin, and adhesion molecules such as VCAM1 [432]. However, studies of fungal ligands for β-1 integrins are far fewer. *In vitro* studies have shown that β-1 integrins on polymorphonuclear leukocytes can bind to the fungal β-glucan, PGG-glucan [423]. Another possibility is that cryptococcal stimulation alters another NK cell surface molecule, which in turn stimulates β-1 integrins in a *cis*- interaction. In this case, knockdown of β-1 integrins would not affect NK-cryptococcal conjugate formations, since β-1 integrins are not binding to a cryptococcal ligand. β-1 integrins have shown the capacity for forming *cis*-interactions [433,434], and NK cell cytotoxicity can regulated by *cis*-interactions [435]. Therefore, future research can be done to explore cryptococcal ligands or *cis*-interactions that are required to activate β-1 integrins.

Previous studies suggest a model of how β-1 integrin signaling leads to Rac1 activation. β-1 integrins mediated activation of ILK is likely through the cytoplasmic portions of their β-chains (reviewed in [436]). ILK then acts as a scaffold protein that contains ankyrin repeats, pleckstrein homology domains, and calponin homology (CH) domains [428]. One class of proteins that interact with ILK are parvins. Parvins are a family of proteins that bind to the CH domain of ILK and are involved in smooth muscle contraction, actin-microtubule attachment, cell polarization, and cell survival [437,438]. Interestingly, β-parvin, has been shown to interact with the guanine nucleotide exchange factor (GEF) α-PIX, which is an activator of Rac1 [439].

In conclusion, β-1 integrin activate a Rac mediated cytotoxicity pathway required for NK cell mediated killing of *Cryptococcus*. The finding that β-1 integrins provide an activation signal rather than enhancing adherence highlights its novel role in fungal killing that is closer to a pathogen associated molecular pattern receptor than an adhesion molecule.

Chapter 5: Role of Arp2/3 and formins on cryptococcal killing

Contributions: Lifeact RFP transfected YT cells were produced by Henry Ogbomo

5.1: Introduction

Actin filaments are involved in numerous cell functions, including cell adhesion, migration, cytokinesis, microtubule trafficking, and survival [440,441]. Actin filaments consist of a leading “barbed” end and a trailing “pointed” end. The nucleation of actin filaments is a key regulatory step in the speed of actin polymerization (reviewed in [340,442]). Actin filament nucleation can be controlled by Arp2/3, formins, and spire [340]. Spire is a newly discovered actin nucleator that lacks evidence of involvement in NK cytotoxicity. This chapter will focus on the role of Arp2/3 and formins on NK anti-cryptococcal activity. Arp2/3 complex consists of 7 subunits (actin-related protein complex (ARPC)1,2,3,4,5, ARP2, and ARP3) that bind to a parent actin filament and resemble the barbed end of an actin filament. New actin monomers are added to this barbed end, and a new actin filament is grown. These new filaments can stay attached to the parent strand and cause actin branching, or the Arp2/3 complex can detach from the parent strand and release the daughter strand in a process called debranching. The branched nature of the actin produced by Arp2/3 gives the actin skeleton structure and

allows resistance to deformation. In NK cell this allows for adhesion to the target and assembly of actin at the immune synapse [443].

Actin filaments can also be nucleated by formins. 15 formins have been identified in humans [444]. Formins bind to actin dimers or trimers and cause elongation of the actin. Elongation of the actin is achieved by formins forming homodimers and developing a donut structure that wraps around the barbed end of actin filaments. This prevents actin capping proteins from attaching to the barbed end and stopping actin growth. The formin homology (FH)1 domain of formins also allows profilin bound actin monomers to be added to the barbed end. Normally, actin monomers that are bound by profilin will not bind to actin filaments. Unlike Arp2/3, loss of the formin - hDia1 - did not reduce the levels of actin at the immune synapse. Instead loss of hDia-1 prevented delivery of cytotoxic granules to the immune synapse due to disruption in MTOC trafficking [445].

Both NK- tumor killing and NK-cryptococcal killing depend on conjugate formation and degranulation of cytotoxic effectors. However, NK cells bind *Cryptococcus* through numerous microvilli, while tumor cell adhere to NK cells through large membrane-membrane contact [335]. This difference in conjugate structure could suggest that different actin remodeling proteins are involved. The smaller contact area of microvilli may not require the large actin plates that form as a result of Arp2/3 activity. Additionally, LFA-1 is not involved in NK-cryptococcal conjugate formation, but is crucial to activating Arp2/3 in tumor cytotoxicity [446]. This raises the possibility that Arp2/3 and formins have different roles in cryptococcal cytotoxicity compared to tumor killing. In this chapter

I investigate the role of Arp2/3 and formins in NK-cryptococcal cytotoxicity.

5.2 Results

NK-cryptococcal conjugates are weaker than NK-tumor conjugates

NK cytotoxicity against cryptococcal target is slower than tumor killing [261,299]. This could be due to a weaker conjugate between NK cell and *C. neoformans*. Using AFM I found that the adhesion force between NK cells and *Cryptococcus* is weaker than the force between NK cells and 721.221 cells (Figure 5-1). There are many factors that can affect the adhesion force. One major component is actin remodeling proteins - Arp2/3 and formins - that create an actin plate and trap receptors at the immune synapse [446]. While both proteins can reorganize actin, Arp2/3 is specific in facilitating conjugate formation [443,446]. Therefore, the weak adhesion between NK cells and the *Cryptococcus* could be caused by a lack of Arp2/3 involvement.

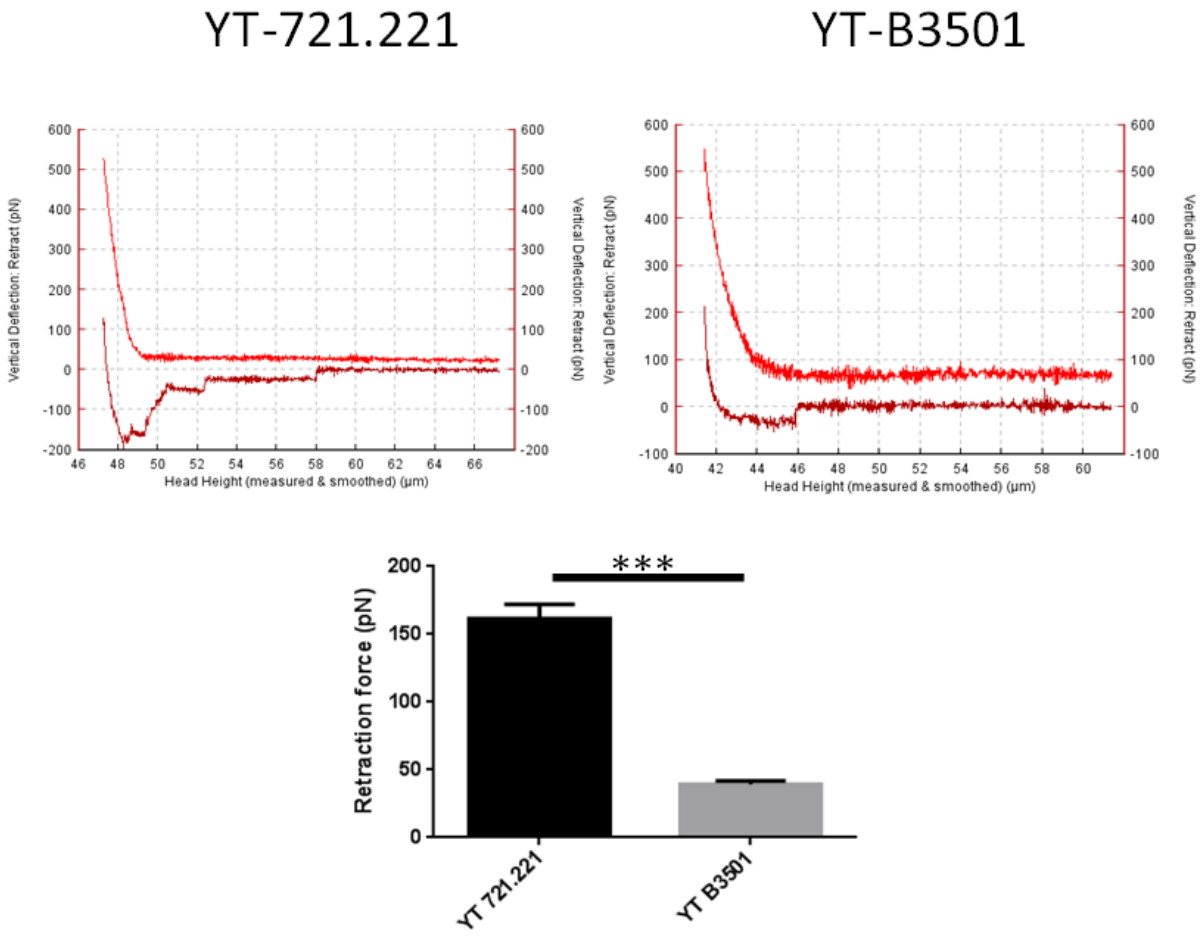


Figure 5-1: Adhesion between *Cryptococcus* and NK cells are weaker than adhesion between 721.221 and NK cells. YT cells were co-cultured with 721.221-CFSE tumor targets or *Cryptococcus* strain B3501 targets in poly-L-Lysine coated dishes. 721.221 or *Cryptococcus* were attached to the cantilever by Cell-Tak. YT cells and the targets were contacted with a force of 500 pN for 10 seconds. A total of 51 contacts between YT and 721.221 and 67 contacts between YT and *Cryptococcus* were recorded. Top) representative extension and retraction curves for the respective conditions. Bottom) statistical analysis of contacts made. Bars represent mean of 3 experiments ± SEM. ***p<0.01.

Formins but not Arp 2/3 is required for NK mediated cryptococcal killing

In order to examine if actin remodeling proteins are involved in cryptococcal cytotoxicity I used the small molecule inhibitors CK666 and SMIFH2 to inhibit Arp2/3 and formins respectively. CK666 binds to the Arp2/3 complex and stabilizes the inactive conformation [447]. SMIFH2 blocks formins associating with the barbed end of actin filaments, and decreases the production of formins [448,449]. I found that CK666 did not affect YT cytotoxicity against *Cryptococcus* (Figure 5-2). However, the same concentrations of CK666 prevented YT cell killing of 721.221 tumor targets (Figure 5-3). This suggests that Arp2/3 is not required for cryptococcal killing, but is required in tumor killing. Unlike Arp2/3, inhibition of formins by SMIFH2 prevented NK cell killing of *Cryptococcus* and 721.221 tumor targets (Figure 5-4). This suggests that formins are required for cryptococcal killing.

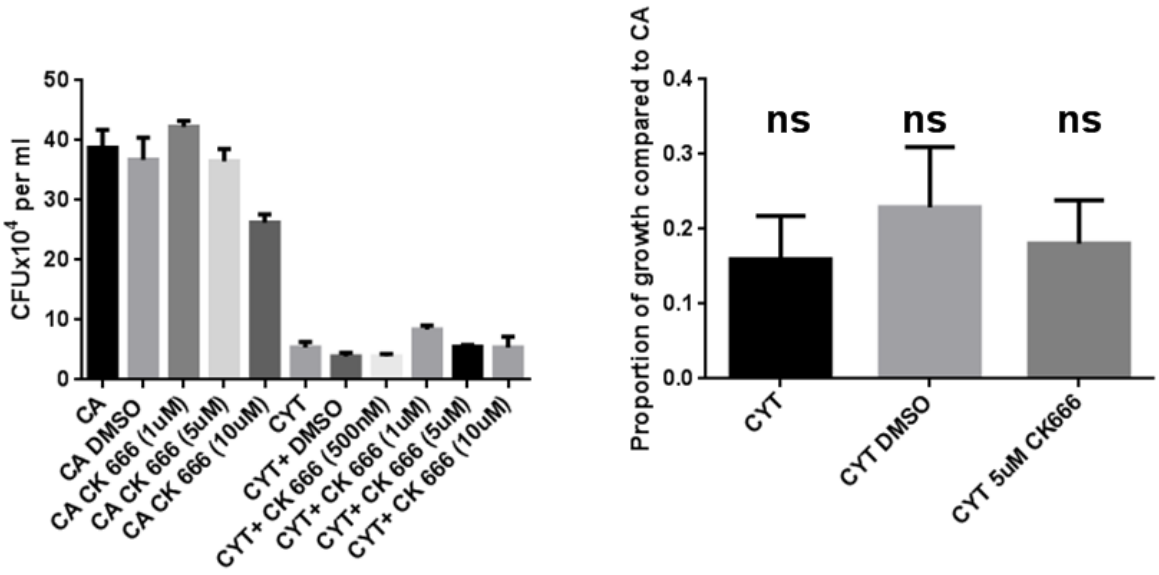


Figure 5-2: Arp2/3 is not required for NK cryptococcal killing. Left: YT cells were co-incubated with *C. neoformans* in the presence of Arp2/3 inhibitor - CK666 - or vehicle control. Bars represent the mean of quadruplicate wells in a single experiment ± SEM. Data is representative of 3 independent experiments. Right: Inter-experimental statistics from 3 experiments. None of the concentrations of CK666 tested significantly inhibited anti-cryptococcal activity.

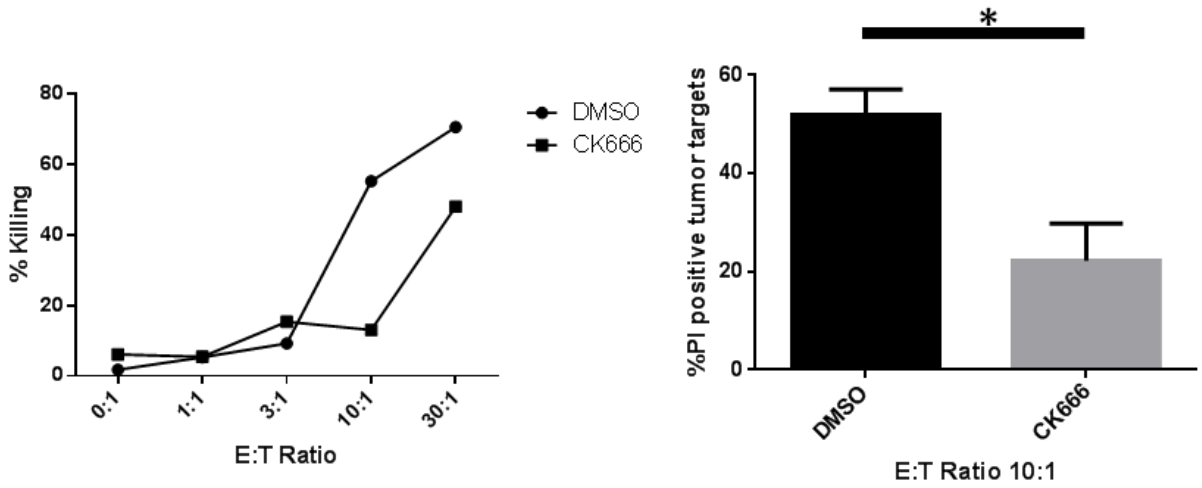


Figure 5-3: Arp2/3 is required for NK tumor killing: Left: YT cells were pretreated 10uM CK666 or vehicle control and then co-cultured with 721.221 tumor targets for 1 hour. Killing was assessed by propidium iodide. Percentage killing = (number of dead tumor targets)/(total number of tumor targets) *100%. Right: Percentage killing at an E:T ratio of 10:1 was pooled from 3 different experiment with 3 different donors. Data was analysed using a T test. Bars represent the mean ± SEM * $p<0.05$

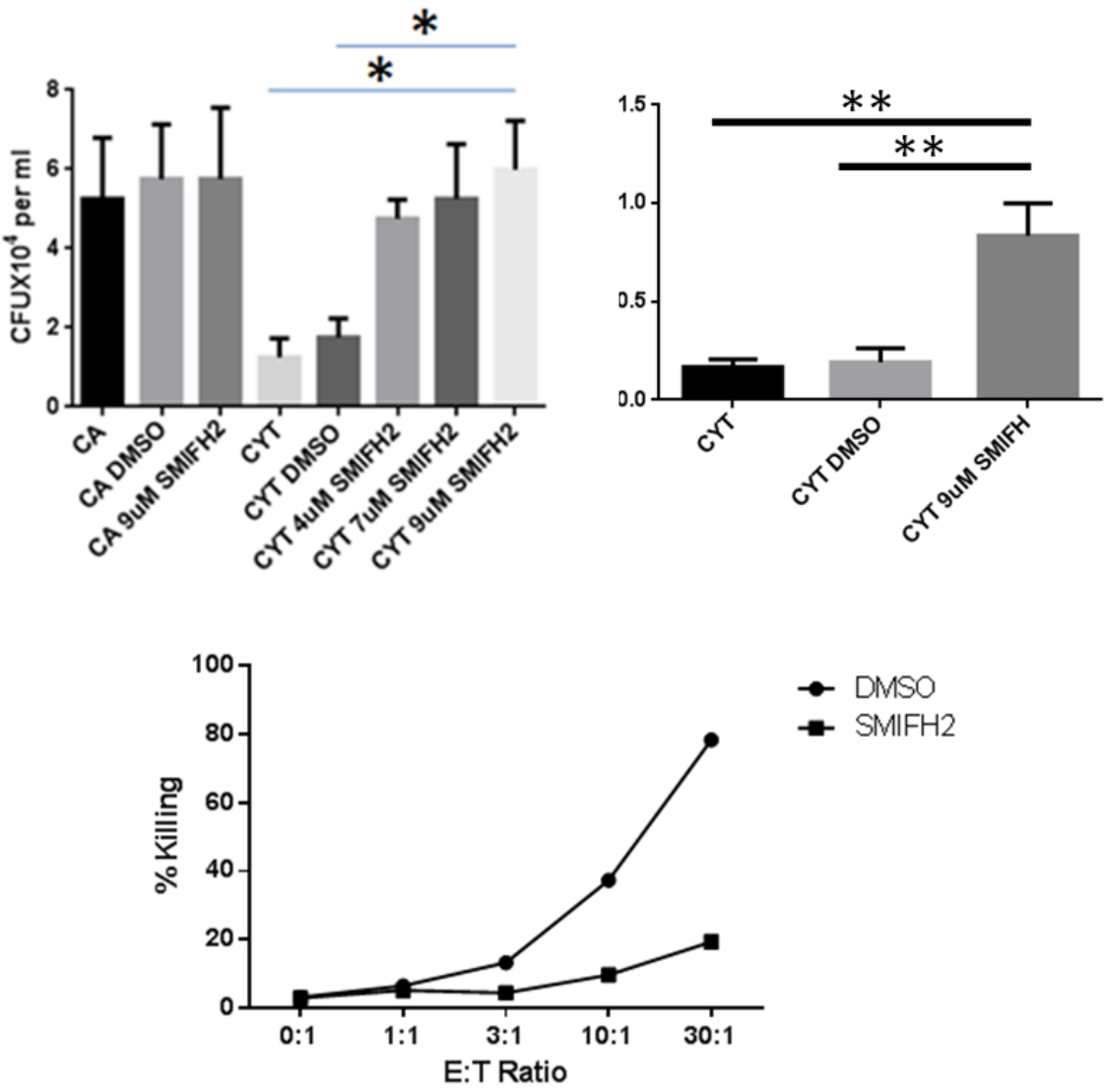


Figure 5-4: Formins are required for NK mediated cryptococcal and tumor killing. Top Left: YT cells were co-cultured with *Cryptococcus* in the presence of the formin inhibitor, SMIFH2. Bars represent the mean of quadruplicate wells in a single experiment ± SEM. Top Right: Inter-experimental statistics from 3 experiments. Bottom: Primary NK cells were preincubated with 3uM SMIFH2. NK and CFSE labelled LCL 721 cells were co-cultured for 1 hours, and then labeled with 7AAD. Killing with assessed by flow cytometry. Percent killing = (number of dead tumor targets)/(total number of tumor targets) * 100%. Data is representative of 3 experiments. * $p<0.05$

Arp2/3 allows tight conjugate formation between NK cells and 721.221

Since Arp2/3 remodels actin at the immune synapse, it likely enhances tumor killing by increasing the adhesion force. In order to determine if the role of Arp2/3 is to increase adherence, I used AFM to test the adhesion force between YT cells and 721.221 targets in the presence of CK666 or vehicle control. Inhibition of Arp2/3 greatly weakened the adhesion force (Figure 5-5), suggesting that Arp2/3 is responsible for the tighter conjugate present in tumor killing. However, inhibition of CK666 did not affect the adhesion force between YT cells and *C. neoformans* (Figure 5-6). Since conjugate formation between YT cells and *Cryptococcus* was not dependent on Arp2/3, I decided to determine if formins are required for adherence.

Formins allows tight cryptococcal and tumor conjugate formation

I measured the adhesion force between *Cryptococcus* strain B3501 and YT cells in the presence of SMIFH2. I found that formins is required for binding between YT cells and *Cryptococcus* (Figure 5-6). I also found that SMIFH2 is required for YT cell binding to 721.221 targets (Figure 5-6). This suggests that NK-tumor binding is dependent on two actin remodeling pathways, while NK-cryptococcal bindings is dependent on only formin pathway.

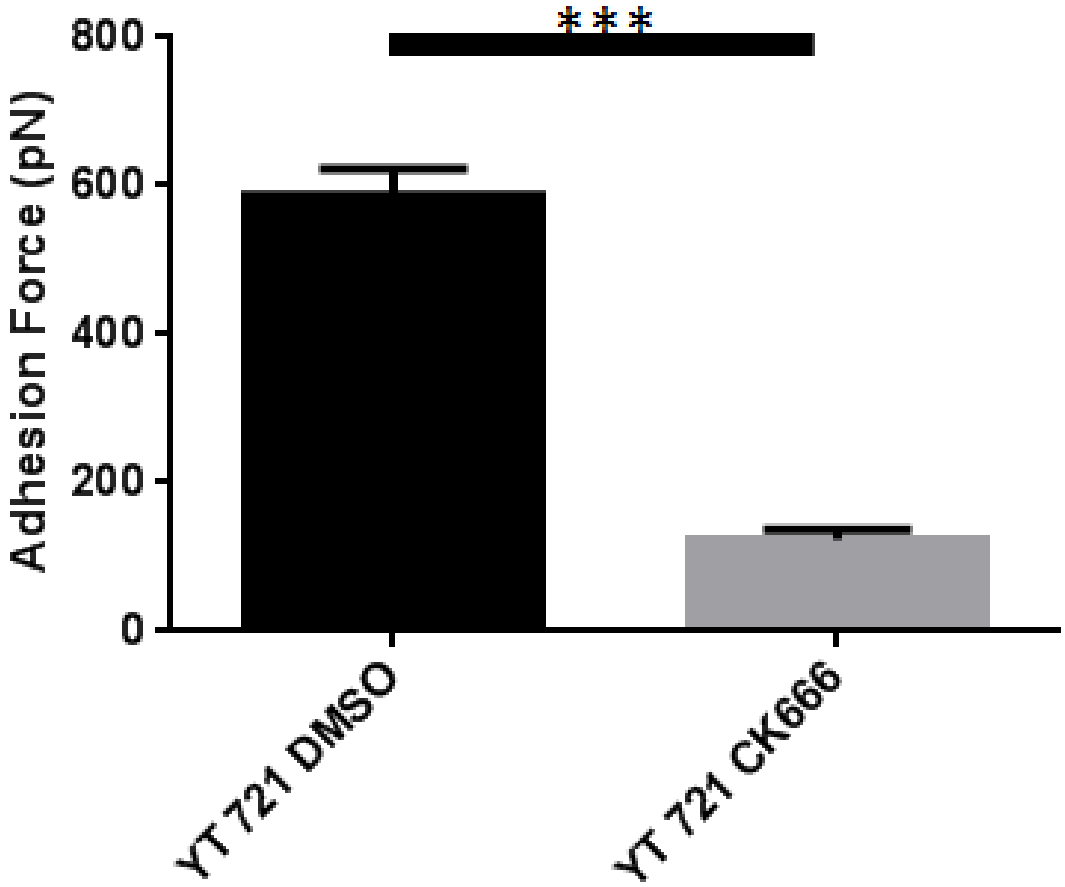


Figure 5-5: Arp2/3 is required for strong conjugate formation between NK cells and tumor targets. YT cells were pre-incubated with DMSO or CK666 and then plated on poly-L-lysine coated dishes. CFSE labelled 721.221 PFA fixed cells were attached to an AFM probe and the adhesion force, after 1 minute of contact, between YT cells and 721.221 cells were measured. Bars indicate average of the adhesion force from 7 cells per condition. Error bars are SEM. Data is representative of 3 experiments. *** $p<0.001$

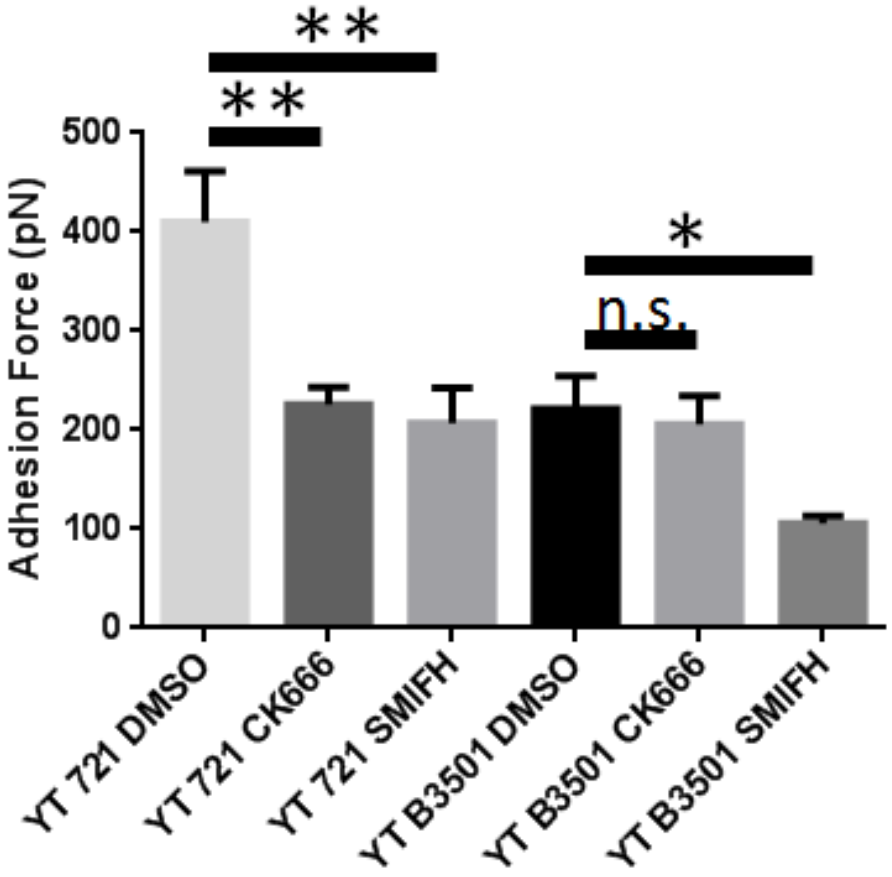


Figure 5-6: Formins are required for strong conjugate formation between both NK cells and tumor targets and NK cells and *Cryptococcus*. YT cells were preincubated with 10uM CK666, 9uM SMIFH2, or DMSO control. 721.221 cells and *Cryptococcus* strain B3501 were fixed with PFA and attached to the cantilever. The adhesion forces between YT cells and tumor or *Cryptococcus*, after 1 minute of contact, were measured. Bars indicate average of the adhesion force from 7 cells per condition. Error bars are SEM. Data is representative of 2 experiments. n.s. not statistically different, * $p < 0.05$, ** $p<0.01$.

Actin accumulates more in NK-tumor conjugates, than in NK-cryptococcal conjugates

The role of Arp2/3 in NK conjugate formation is to facilitate actin polymerization at the immune synapse [443]. Therefore, I expected that the degree of actin polymerization at the synapse between *Cryptococcus* and NK cells is less than in a tumor synapse, and that CK666 would reduced actin plate formation between YT cells and 721.221 targets. YT Lifeact-RFP cells, with RFP labeled actin structures, were co-cultured with 721.221 cells or *Cryptococcus* in the presence of CK 666 or vehicle control. I found that the immune synapse between YT and 721.221 contained higher levels of actin compared to the cryptococcal synapse (Figure 5-7). This complements the previous findings of a tighter NK conjugate with tumor targets than *Cryptococcus*. Additionally, actin polymerization at the IS was inhibited with the addition of CK666, suggesting that the accumulation of actin at the NK-tumor synapse is Arp2/3 dependent. This is consistent with the canonical model of immune synapse formation [443,450].

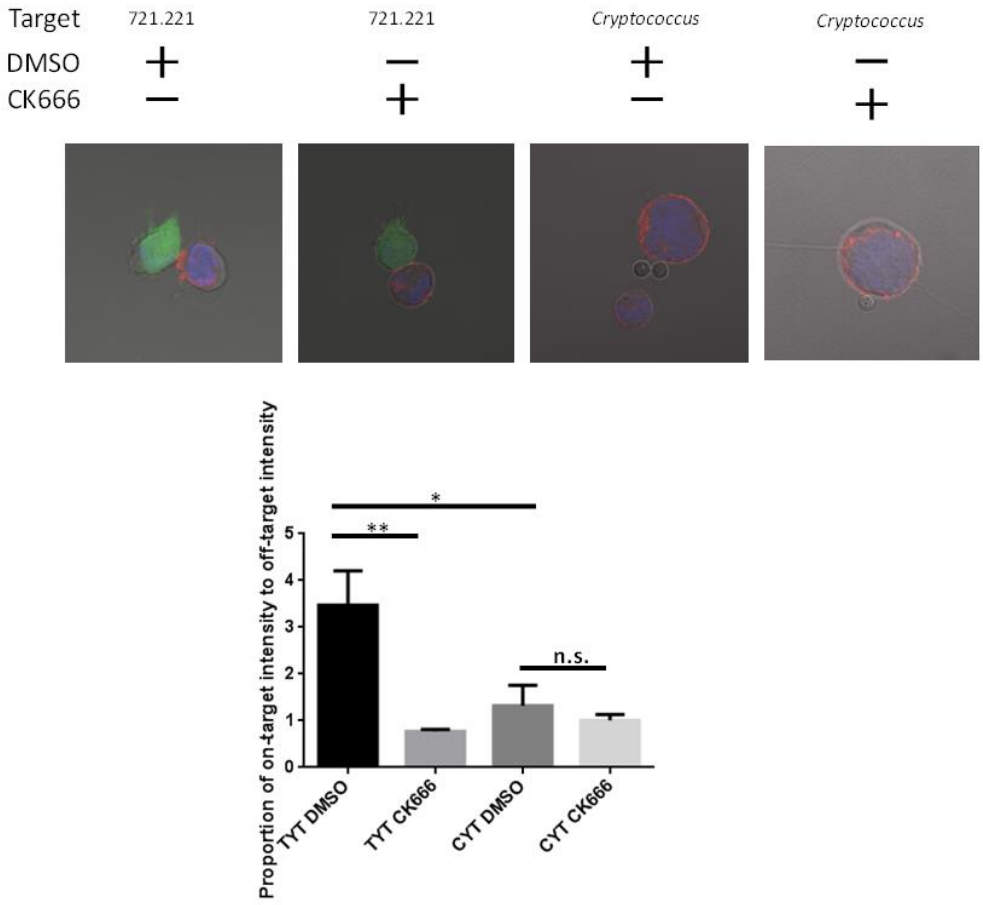


Figure 5-7: NK cells increase actin at the immune synapse against tumor targets, through an Arp2/3 dependent mechanism. YT cells transfected with LifeAct - RFP were co-cultured with 721.221 tumor cells or *Cryptococcus* in the presence of CK666 or vehicle control. Top: Representative images of YT cells in contact with their targets in the different conditions. Red is actin, green is CFSE labelled 721.221, and blue is nuclear staining. Bottom: Statistical analysis of actin polarization in YT cells. The site of contact was set as the center of the interface between the tumor target or cryptococcal target and the YT cell. For analysis a line was drawn from the site of contact to the center of the YT cells. Actin that deviated by 35 degrees or less from this line was considered on-target, while actin that deviated between 35-70 degrees was considered off-target. The ratio of on-target actin to off-target actin was calculated by (intensity of on-target actin)/(intensity of off-target actin). A minimum of 5 conjugates were analyzed per condition. Bar represent the mean of at least 5 conjugates in a single experiment ± SEM. Data is representative of 3 experiments. n.s. not statistically different * $p < 0.05$, ** $p < 0.01$.

5.3 Discussion

In this chapter I showed that: i) the adhesion force between NK cells and *Cryptococcus* is weaker than the adhesion force between NK cells and tumor targets, ii) actin remodeling proteins - Arp2/3 and formins- are involved in NK cell mediated tumor killing, but only formins are required for cryptococcal killing, iii) Arp2/3 activity is responsible for the stronger adhesion force between NK cells and tumor targets, iv) Formins are required for NK binding to *Cryptococcus*, iv) Arp2/3 is also responsible for increased actin polymerization at the immune synapse between NK cells and tumor targets,

Initialization of the NK lytic synapse is dependent on actin branching caused by Arp2/3, which allows clustering of activating receptors at the immune synapse [451]. My data show that Arp2/3 is not involved in NK-cryptococcal cytotoxicity and resulted in reduced actin polymerization at the site of contact, which could have prevented the clustering of anti-cryptococcal receptors and partially explains the weak adhesion force. These data complement previous studies that showed the NK-cryptococcal synapse is weaker than the NK-tumor synapse [335]. Although the exact cause for the absence of Arp2/3 involvement is unclear, NK cells depend on LFA-1 signaling in order to activate Arp2/3 [446]. Since LFA-1 is not required in NK cell-cryptococcal killing, this could explain the lack of Arp2/3 involvement.

In this chapter I found that formins were involved in NK binding to *Cryptococcus* and anti-cryptococcal cytotoxicity, which suggests that the loss of cytotoxicity is due to a reduction in adhesion. However, mechanism of formins in adhesion is unclear. One

possibility is that formins promote the formation of microvilli, which are required for NK cell interaction with *Cryptococcus* [452]. The formation of microvilli on lymphocytes has also been shown to be an WASP-Arp2/3 independent process [453], which would explain why inhibition of Arp2/3 does not reduce NK adhesion to *Cryptococcus*. Alternatively, formins and Arp2/3 have been shown to be involved NK adherence to matrix protein, suggesting that formins may also be involved in receptor trapping and immune synapse formation [443]. Additional studies will be required to determine the exact role of formins in NK binding of *C. neoformans*.

Formins involvement in cytotoxicity could be to its role in adherence. However, formins can also contribute to cytotoxicity is through their role in microtubule rearrangement and polarization of the MTOC to the IS [344,454]. The loss of formins in NK cells have impairs degranulation [443]. Since granule trafficking is dependent on the microtubule network, formins could be activated in cryptococcal killing in order move the MTOC closer to the immune synapse and allow for targeted granule exocytosis.

Additional studies are required to determine how formins are activated in cryptococcal killing, however Rac1 could be a good candidate. Rac1 is required for the activation of the PI3K → Erk anti-cryptococcal cascade [263]. Rac1 is also able to bind to the formin, Formin Homology 2 Domain containing 1 (FHOD1), which then recruits FHOD1 to actin networks and facilitates actin polymerization [455].

The weak adhesion between NK cells and *Cryptococcus* suggests that the interaction

between the two cells is more transient. It is also known that NK cell killing of *Cryptococcus* is slower than with tumor targets [299]. This raises the possibility that the slower cytotoxicity is due to the transient nature of the cryptococcal synapse. This suggests that NK cells either require multiple contacts with *Cryptococcus* in order to meet a threshold level of activation. However, alternative explanation also exist, such as a single contact initiates a cytotoxic mechanism that requires a prolonged period of time to facilitate killing. Additional studies into these possibilities could help further tease apart the differences between NK mediated anti-tumor and anti-microbial activity.

In summary, my data shows that that NK cell adhesion to *Cryptococcus* and tumor targets is different. This difference is likely caused by reduced actin polymerization at the synapse due to a lack of Arp2/3 involvement. While actin remodeling at the immune synapse is different, other roles of actin are maintained by formins - such as MTOC polarization. The lack of Arp2/3 helps address the higher E:T ratio and longer incubation time required to achieve anti-fungal killing.

Chapter 6: Discussion

My data has shown that anti-cryptococcal cytotoxicity in NK cells requires 1) β-1 integrin activation of an ILK→ Rac1 pathway, 2) Rac1 cooperation with SFK to activate a PI3K → Erk pathway, and 3) formin activity, but not arp2/3, for NK cryptococcal killing. These findings contribute to our understanding of NK cell biology and signal transduction.

My data expands our knowledge of integrins in microbial host defense. Evidence of β-1 integrin involvement in fungal killing was limited. One study demonstrated the ability of β-1 integrin antagonists blocking the activation of neutrophils by PGG glucans [423]. My data shows that β-1 integrins are involved in protection against live fungal pathogens, and that the anti-fungal capacity of β-1 integrins in not limited to neutrophils. My data also suggests that β-1 integrins are not acting as adhesion receptors and are not involved in the affinity of NK cells for *C. neoformans*. Rather β-1 integrins are functioning similar to activation receptors on NK cells by initiating cytotoxic signaling.

My findings also explore the role of NK cytotoxicity in anti-fungal killing. My studies demonstrate that NK cells possess an anti-fungal killing mechanism that differs significantly from the established model of tumor and anti-viral killing. NK anti-fungal killing utilizes β-1 integrins instead of β-2. The role of Rac1 is different from its function in tumor cytotoxicity. My data also shows that NK cell adhesion to tumor targets is stronger than toward *Cryptococcus*. This is partly due to a lack of Arp2/3 dependent

actin remodeling. However, despite the reduced actin remodeling at the IS and weak conjugate adhesion force, NK cells are still capable of killing *Cryptococcus*. One explanation is that NK cells possess an actin independent mechanism of conjugate formation. The actin plate is normally crucial in organizing NK activating receptor and adhesion molecules into a peripheral supramolecular activation complex (pSMAC) and central supramolecular activation complex (cSMAC) [296]. The lack of actin remodeling suggests that the canonical pSMAC and cSMAC may not develop in anti-microbial killing. However, despite reduced actin plate formation, polarization of perforin containing granules to the IS still occurs [456]. This suggests that Arp2/3 is not required for NK cell polarization against fungal targets. This is unlike polarization during leukocyte migration, which is Arp2/3 dependent [457].

NK cell anti-tumor killing is highly dependent on β-2 integrins - specifically LFA-1. LFA-1 provides both adherence to the target, as well as activating cytotoxic signaling pathways. My findings showed that β-1 integrins were required for anti-cryptococcal activity. However, they did not increase adherence. Instead β-1 integrins activated Rac1 signaling. My data also shows that Rac1 signals together with NKp30→ SFK to initiate degranulation and cytotoxicity. This data demonstrates a model of receptor cooperation similar to ADCC by NK, where integrin - LFA-1 - signaling caused granule polarization towards the IS and CD16 signaling resulted in degranulation [10]. Additional studies will be required to determine if β-1 integrins cause granule polarization to the IS in a manner similar to LFA-1.

My data also supports a model of Rac activating PI3K, and complements *in vitro* studies that showed that Rac could bind to the p110 β subunit of PI3K and cause PI3K activation [408]. This activation of PI3K was partly dependent on its p85 regulatory subunit, because a less inhibitory p85 subunit enhanced Rac dependent PI3K activity [408]. This dependency on both the p85 subunit and Rac binding for PI3K activation is consistent with previous studies that demonstrated that the p85 subunit of PI3K is also necessary for cryptococcal killing [284].

Neutrophil studies have found that Rac1 and PI3K can activate each other in a positive feedback loop [392]. Although, NK cell signaling is slightly different since I have demonstrated that Rac1 activation of PI3K in cryptococcal killing is non-reciprocal, whereas in NK cell mediated tumor killing, Rac1 is activated by PI3K [458]. It is known that different stimuli can activate different signaling pathways. However, my data shows that different stimuli can activate the same proteins, but the proteins can serve different functions. It is unclear why Rac swaps between an effector and an activator of PI3K, but a likely answer involves the influence of other signaling proteins that are also activated by *Cryptococcus*. The finding that Rac can both be an activator and effector of PI3K highlights the plasticity of signaling molecules and the importance of testing the function of signaling proteins in the specific system they are used.

The two anti-cryptococcal receptors identified - NKp30 and β-1 integrins - appear to contribute the cytotoxicity by activated the PI3K→ Erk cascade [9]. Inhibition of PI3K → Erk signaling results in reduced perforin polarization towards the IS, and reduced

perforin degranulation [284]. This is in contrast to NK ADCC, where FcR signaling controls granule fusion with the plasma membrane, and integrin signaling controls polarization of granules towards the IS [10]. The key difference is that cryptococcal signaling converges on a single (PI3K → Erk) pathway that controls both perforin polarization and degranulation, while in ADCC, receptor signaling is kept separate and each pathway is responsible for either perforin polarization or degranulation but not both.

Perforin degranulation also depends on actin remodeling at the IS. In order for degranulation to occur the actin plate needs to be reorganized so that there are conduits through which granules can dock and fuse with the plasma membrane [296]. This reorganization normally depends on Arp2/3 is conjunction with proteins that depolymerize actin such as cofilin and coronin 1A [459,460]. Since Arp2/3 is not involved in cryptococcal killing, it is unclear if there is another redundant protein responsible for forming conduits in the actin plate. Alternatively, reduced actin polymerization at the cryptococcal IS may allow granule fusion without the need for actin reorganization.

My research also has implication for other leukocytes. β-1 integrins are ubiquitously expressed in most cell types. It is unclear if β-1 integrins in other leukocytes also recognize *Cryptococcus* and initiate signaling. CD4 T cells from AIDS patients have elevated levels of α5 β1 integrins [461]. Since *Cryptococcus* is associated with HIV infections and CD4 T cells are directly cytotoxic to *C. neoformans* [162], additional studies can be done to investigate if increase α5β1 on CD4 T cells enhances or hinders

cytotoxicity.

My data also identified targets of immunotherapy against *C. neoformans*. Both cell permeable and receptor driven small molecule activators of Rac1 exist and could enhance NK anti-cryptococcal activity [462,463]. Bispecific antibodies that recognize the β chain of β-1 integrins and cryptococcal capsular antigen could enhance β-1 integrin signaling and lead to improved cryptococcal clearance. Activating antibodies against β-1 integrins may also enhance NK-cryptococcal killing. Since Arp2/3 plays a vital role in NK-tumor killing, an exogenous activator of Arp2/3 could enhance conjugate formation with *Cryptococcus*. While there is no direct pharmacologic activator of Arp2/3, Arp2/3 activity is regulated by Cdc42, which has potent activators [464]. β-2 integrins, specifically LFA-1, in tumor killing provide NK cells with strong cytotoxic signals [303], therefore crosslinking LFA-1 with bi-specific antibodies to GXM may enhance anti-cryptococcal activity. Alternatively, β-1 integrin stimulation could help enhance anti-tumor cytotoxicity. LFA-1 dominates as to primary integrin in tumor killing, however bi-specific antibodies to tumor specific surface antigen and β-1 integrins could augment NK cell tumor killing.

There are still many questions that remain to be answered in the field of NK cell mediated anti-microbial killing. The weak adhesion force between NK cells and *Cryptococcus* suggests that NK cells have difficulty maintaining a proper IS. It is unclear if NK cells utilize another mechanism to kill at a distance, or if there is a strong chemotactic gradient that keeps the NK cell in close proximity to its target. The

cryptococcal ligand that activate β-1 integrins is also unknown. Since β-1 integrins can bind to certain forms of β-glucans [423], examining if β-1 integrins bind to beads coated with cryptococcal β-glucans can prove enlightening. In human ovarian cancer cells α5β1 integrins activates the formin FHOD3 and allows of arp2/3 independent migration [465]. It would be tantalizing to test if the formin pathway is activated by β-1 integrins in NK cell cryptococcal killing. Additional experiments are also needed to investigate if enhanced NK cell anti-cryptococcal activity alone is sufficient to alter the course of *C. neoformans* infection in humans.

In conclusion, my thesis demonstrates the differences between NK cell anti-fungal killing and anti-tumor killing. It highlights the plasticity of signaling pathways that can be activated by similar receptor stimulation. My findings expand the anti-microbial role of NK cells, but also leaves additional questions to explore.

Chapter Appendix A: Source code for analysis of microscopy images

```
autoAnalyzeData = function(instruct_filename){
  #This function will automatically read a .csv file and analyze all the images in that file


  #Constants
  channel_int = 1
  intensity_threshold = 0.15
  minimal_distance = 0

  #import the .csv instructions file
  instructionTable = read.csv(instruct_filename)
  numOfRows = dim(instructionTable)[1] #this is the number of entries in the table
  print(numOfRows)

  for(i in seq(1,numOfRows,1)){
        Image_Dir = instructionTable[i,"Image.DirTable"]
        Image_Name = instructionTable[i,"Image.NameTable"]
        Image_Name = unname(Image_Name)
        print(toString(Image_Name))
        point_x = instructionTable[i,"IS.X"]
        point_y = instructionTable[i,"IS.Y"]
        center_x = instructionTable[i,"Center.X"]
        center_y = instructionTable[i,"Center.Y"]

                        intensityFromPointWithCircumference(Image_Dir,Image_Name,poi
      nt_x,point_y,center_x,center_y,channel_int,intensity_threshold,minimal_distance)
```

```
  }
}

intensityFromPointWithCircumference =
function(Image_Dir,Image_Name,point_x,point_y,center_x,center_y,channel_int,intensit
y_threshold,minimal_distance){
  #This function is to determine the intensity of all the point of a certain channel and their
distance from a designated point

  #calculation constants
  #this it angle angle a pixel has to be to be consider at the IS
  ANGLE_FOR_ON_TARGET = 35
  #if a pixel is between the on-target and this value, then it is considered off-target
  ANGLE_FOR_OFF_TARGET = 70

  OnTargetIntensity =0
  OnTargetCount = 0
  OffTargetIntensity =0
  OffTargetCount = 0

  #DEBUG control variable
  DEBUG=TRUE
  scale_Line_X = 10
  scale_Line_Y = 10
  scale_Line_Length = 10

  #Close to IS distance
  CLOSE_TO_IS = 30
  num_Pixel_CTI =0
  total_Num_Pixel =0
  fluor_CTI =0
  total_Fluor =0

  Image_Path = paste(Image_Dir,Image_Name,sep="")
  Image = readImage(Image_Path)

  if(channel_int == 1){
        #want to analyze the red channel
        channel_Image = channel(Image,"asRed")
```

```
}
else if (channel_int == 2){
	#want to analyze the green channel
	channel_Image = channel(Image,"asGreen")
}
else if (channel_int == 3){
	#want to analyze the blue channel
	channel_Image = channel(Image,"asBlue")
}
#display the original image for comparison
display(Image)

#display the image in the channel requested
display(channel_Image)
temp_path = paste(Image_Dir,"Channel Image.tiff")
writeImage(channel_Image,temp_path,quality=100,bits.per.sample = 16)

#determine the dimensions of the image
image_Dim = dim(channel_Image)

#store the image data in a usable variable
channel_Image_Data = imageData(channel_Image)

#if DEBUG create a matrix to store the points that are chosen
points_Chosen_Matrix = channel_Image_Data

#create a matrix that will store the distances of different points to the immune synapse
distance_Matrix = matrix(,image_Dim[1],image_Dim[2])

#create a matrix that will store the intensity of different points to the immune synapse
intensity_Matrix = matrix(,image_Dim[1],image_Dim[2])

#create a matric the will store the angle of the point of interest from the
angle_Matrix = matrix(,image_Dim[1],image_Dim[2])

#the distance between the center and the IS
dist_IS_To_Center = sqrt((point_x-center_x)^2+(point_y-center_y)^2)

for(i in seq(1,image_Dim[1],1)){
	#for all rows
```

```
for(j in seq(1,image_Dim[2],1)){
#for all columns

#if DEBUG
if(DEBUG){
#Create an image showning which points were chosen (above threshold)
points_Chosen_Matrix[i,j] = 0

}

if(channel_Image_Data[i,j]>=intensity_threshold){
#if the current point is bright enough

#calculate the distance of the point of interest to the center, will be used to determine the angle and if the point is far enough away from center to be consider circumferential
dist_Point_To_Center = sqrt((i-center_x)^2+(j-center_y)^2)

if (dist_Point_To_Center > minimal_distance){
#calculate the distance to the IS
distance_Matrix[i,j] = sqrt((i-point_x)^2+(j-point_y)^2)


dist_To_Center = dist_Point_To_Center

#calculate the angle made by a line from the current point to the center and then from center to IS
dist_To_IS = distance_Matrix[i,j]
angle_Matrix[i,j] = 180/pi*acos((dist_To_Center^2+dist_IS_To_Center^2-dist_To_IS^2)/(2*dist_To_Center*dist_IS_To_Center))


if(is.finite(angle_Matrix[i,j])==FALSE){
error_value = (dist_To_Center^2+dist_IS_To_Center^2-dist_To_IS^2)/(2*dist_To_Center*dist_IS_To_Center)
cat('NaN detected, ', error_value,' \n' )
cat('dist to IS is, ', dist_To_IS ,' \n')
cat('i: ',i,'\n')
cat('j: ',j,'\n')
if(error_value < -1){
```

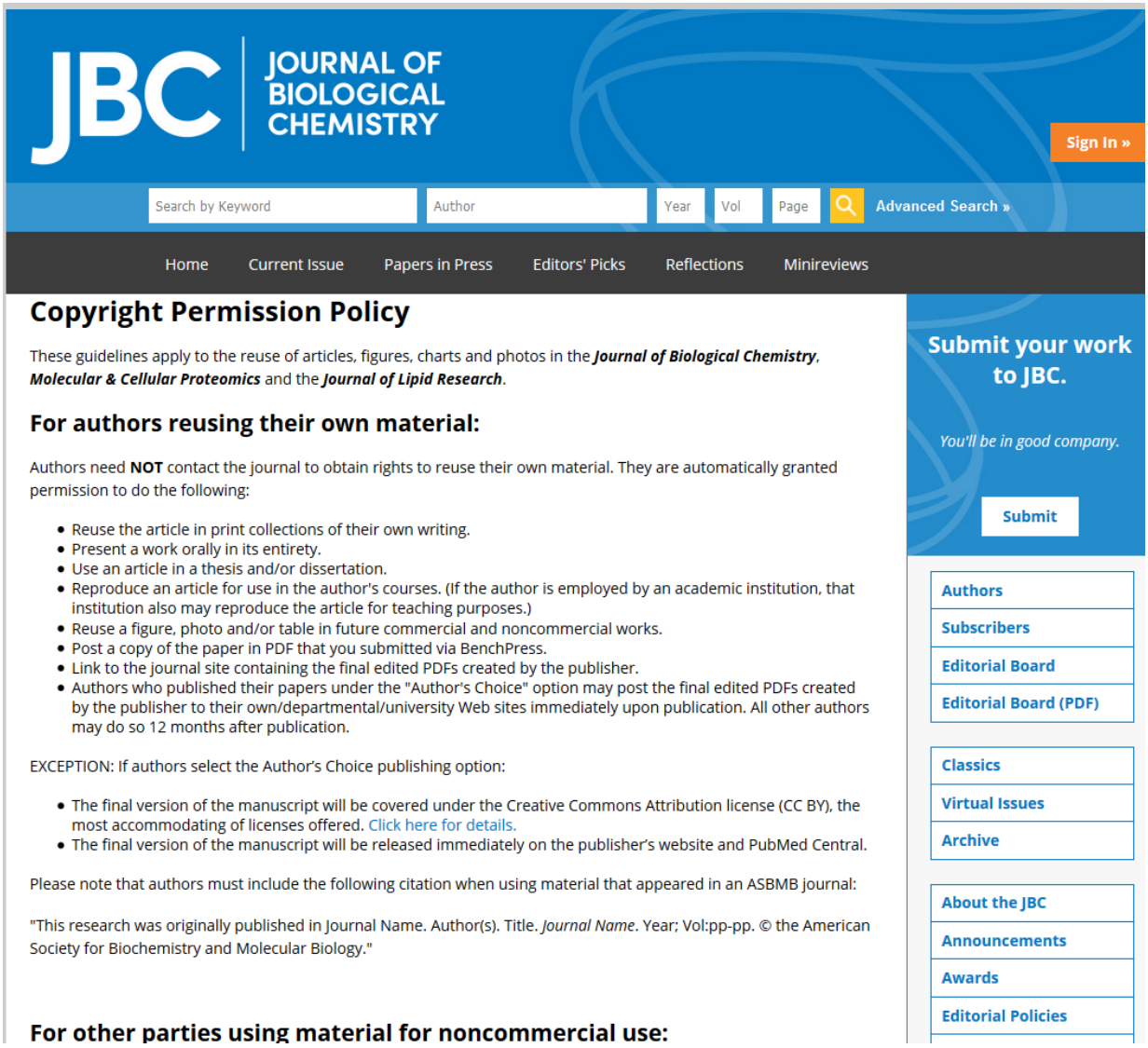
```
        angle_Matrix[i,j] = 180
        }
        else if(error_value > 1){
        angle_Matrix[i,j] = 0
        }
        }


        #determine if the pixel is considered on target or off target or irrelavent
        if(angle_Matrix[i,j] <= ANGLE_FOR_ON_TARGET){ #pixel is considered at the
IS
        OnTargetIntensity = OnTargetIntensity + channel_Image_Data[i,j]
        OnTargetCount = OnTargetCount + 1
        }
        else if(angle_Matrix[i,j] <= ANGLE_FOR_OFF_TARGET){ #Pixel is consider off
target
        OffTargetIntensity = OffTargetIntensity + channel_Image_Data[i,j]
        OffTargetCount = OffTargetCount + 1
        }




        #calculate the intensity to the point of interest
        intensity_Matrix[i,j] = channel_Image_Data[i,j]

        #If DEBUG create an image showning which points were chosen
        points_Chosen_Matrix[i,j] = 1

        #if this pixel is close to the IS
        if(distance_Matrix[i,j] <= CLOSE_TO_IS){
        num_Pixel_CTI = num_Pixel_CTI + 1
        fluor_CTI = fluor_CTI + intensity_Matrix[i,j]
        }
        #increase the total number of pixel and the cumulative fluorescence
        total_Num_Pixel = total_Num_Pixel+ 1
        total_Fluor = total_Fluor+ intensity_Matrix[i,j]
        }
```

```
        }



        #draw the scale line if DEBUG and indicate where the IS is
        if(DEBUG){
        if(i >= scale_Line_X && i<= scale_Line_X + scale_Line_Length && j ==
scale_Line_Y){
        Image[i,j,1] = 1
        Image[i,j,2] = 1
        Image[i,j,3] = 1
        }

        if(i==point_x && j == point_y){
        Image[i,j,3] = 1
        }



        }




        }

 }

 if(DEBUG){
        # display the image with scale bar and IS
        #display the points chosen image
        #gob = ebimageGrob(Image,raster = TRUE,x=0.2,y=0.7,scale = 0.85)
        #gob2 = ebimageGrob(channel_Image,raster = TRUE,x=0.7,y=0.7,scale = 0.85)
        #grid.draw(gob)
        #grid.draw(gob2)

        display(Image)
        temp_path = paste(Image_Dir,"Analyzed Image.tiff")
        writeImage(Image,temp_path,quality=100,bits.per.sample = 16)

        display(points_Chosen_Matrix)
        temp_path = paste(Image_Dir,"Points Chosen.tiff")
```

```
        writeImage(points_Chosen_Matrix,temp_path,quality=100,bits.per.sample = 16)


  }


  #vectorize the x and y matrixes
  x=c(angle_Matrix)
  y=c(intensity_Matrix)

  #remove NA values from x and y matrixes
  x = na.omit(x)
  y = na.omit(y)

  #convert the pixel distances to microns (1 um/pixel if Bin 1x1)
  x = x*1

  #plot intensity on the vertical axis and distance to point of interest on the horizontal
axis

  temp_path = paste(Image_Dir,"Intensity Plot.jpg")
  jpeg(temp_path)
  plot(x,y,main=" ", xlab="Angle of pixel from IS (degrees)", ylab="Intensity of pixel",
ylim=c(0,1), xlim=as.integer(c(0,250)))
#as.integer(c(0,sqrt(image_Dim[1]^2+image_Dim[2]^2)))/10, )

  graphics.off()


  #Add blue Lowess line
  #lines(lowess(x,y), col="blue")

  #Add Red linear regression line
  #abline(lm(y~x), col="red")

  #draw a histogram with the number of points above threshold on veritcal axis and
distance from IS on horizontal axis
  hist(x, breaks=20,col="red", xlab ="angle of pixels from IS (degrees)", ylab = "Number
of pixels above the threshold",xlim=as.integer(c(0,250)))
#as.integer(c(0,sqrt(image_Dim[1]^2+image_Dim[2]^2)))/10)
```

```
  #detach from further plotting on the same graph



  #draw a kernal density plot with the number of points above threshold on vertical axis
and distance from IS on horizaontal axis
  #density_Plot = density(x)
  #plot(density_Plot,main="Kernal Density Plot of distance of pixels from IS")
  #polygon(density_Plot, col = "blue", border = "red")

  ratio_on_to_off = OnTargetIntensity/OffTargetIntensity
  avg_on_intensity = OnTargetIntensity/OnTargetCount
  avg_off_intensity = OffTargetIntensity/OffTargetCount
  ratio_on_to_off_avg_intensity = avg_on_intensity/avg_off_intensity
  Image_Name = toString(Image_Name)

  temp_path = paste(Image_Dir,"Data.txt")
  fileConn<-file(temp_path)
  writeLines(c("Filename: ", Image_Name,"IS x:",point_x,"IS y:",point_y,"Center
x:",center_x,"Center y:",center_y,"Ratio on target to off target: ",ratio_on_to_off,"Ratio
average on target to off target intensity: ",ratio_on_to_off_avg_intensity), fileConn)

  close(fileConn)

  #display information about fluorescent and number of pixels close to IS
  #cat('Pixels that are within ', CLOSE_TO_IS,' pixels of the IS are considered close to
the IS \n' )
  #cat('There were ', num_Pixel_CTI,' fluorescent pixels close to the IS \n')
  #cat('There were ',total_Num_Pixel,' fluorescent pixels analyzed in the image \n')
  #cat('',num_Pixel_CTI/total_Num_Pixel*100,'% of fluorescent pixels were close to the
IS \n')
  #cat('',fluor_CTI/total_Fluor*100,'% of fluorescence is close to the IS')

}
```

Chapter Appendix B: Copyright Documentation



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