IL-12 Restores Defective NK Cell Anticryptococcal Activity in HIV-Infected Patients

by

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Publications

Contributions

This thesis has been published in the following article:

“The NK receptor NKp30 mediates direct fungal recognition and killing and is diminished in NK cells from HIV-infected patients.”


I designed, and performed all experiments on the samples from HIV-infected patients and contributed figures to this article.

2. Part of this thesis is a manuscript in progress entitled “Mechanisms by which IL-12 corrects defective NK cell anti-cryptococcal activity in HIV-infected patients”


Shaunna M. Huston, Pina Colarusso, M. John Gill, and Christopher H. Mody.

All the experiments were designed and performed by myself. I performed all the data analysis and completed all the figures.

I wrote the original manuscript made changes to the manuscript under the supervision of Christopher H. Mody.
Abstract

*Cryptococcus neoformans* is a pathogenic yeast that causes life-threatening pneumonia and meningitis in AIDS patients. Natural killer (NK) cells are important effector cells that directly recognize and kill *C. neoformans* via perforin-dependent cytotoxicity. Although it had previously been demonstrated that, NK cells from HIV-infected patients have defective anticryptococcal activity, and IL-12 restored the activity, almost nothing was known of the mechanisms causing this defect or how IL-12 restored the defect. By examining the sequential steps in NK cell killing of *Cryptococcus*, I determined that NK cells from HIV-infected patients had lower intracellular perforin expression, defective perforin release and defective ability to polarize perforin to the fungal synapse. Importantly, treatment of NK cells from HIV-infected patients with IL-12 reversed these defects and restored the defective anticryptococcal activity. Thus, there are multiple defects in the cytolytic machinery of NK cells from HIV-infected patients, which result in the defective anticryptococcal activity, and these defects can be reversed with IL-12.
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Finally I would like to thank my family for their encouragement and support.
Dedication

This thesis is dedicated to my grandfather, James Adu. Grandpa, your unflinching support for me and staunch perseverance keep pushing me to greater heights. Rest in peace eternally in the land of shadows.
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<th>Description</th>
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<tr>
<td>ADCC</td>
<td>Antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>ART</td>
<td>Antiretroviral therapy</td>
</tr>
<tr>
<td>Asialo-GM1</td>
<td>Ganglio-N-tetraosylceramide</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T-lymphocytes</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
</tr>
<tr>
<td>E:T</td>
<td>Effector to target</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated DD-containing protein</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>GXM</td>
<td>Glucuronoxylomannan</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNKR</td>
<td>Inhibitory natural killer receptors</td>
</tr>
<tr>
<td>IS</td>
<td>Immunological synapse</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>KIR</td>
<td>Killer inhibitory receptor</td>
</tr>
<tr>
<td>LAMP-1</td>
<td>Lysosomal associated membrane protein -1</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Lymphocyte function associated antigen-1</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MACPF</td>
<td>Membrane-attack complex/perforin domain</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility complex</td>
</tr>
<tr>
<td>MTOC</td>
<td>Microtubule-organizing center</td>
</tr>
<tr>
<td>NCR</td>
<td>Natural cytotoxic receptor</td>
</tr>
<tr>
<td>NWN</td>
<td>Nylon wool-nonadherent</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3 kinase</td>
</tr>
<tr>
<td>PMNL</td>
<td>Polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>Src</td>
<td>Sarcoma viral oncogene homology</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>WASp</td>
<td>Wiskott-Alderich syndrome protein</td>
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CHAPTER ONE: INTRODUCTION

1.1 Epidemiology of Cryptococcus neoformans

*Cryptococcus neoformans* is a pathogenic encapsulated yeast that is a major cause of fungal pneumonia, meningitis and meningoencephalitis especially in immunocompromised patients (1, 2). There are more than 36 known species of the genus *Cryptococcus*. Two of these species are considered important human pathogens; *C. neoformans* and *C. gattii*. *C. neoformans* generally causes disease in immunocompromised patients whereas *C. gattii* is more common in immunocompetent patients (3, 4). *C. neoformans* var. *grubii* is the most prevalent species and distributed worldwide, whereas *C. neoformans* var. *neoformans* is found predominantly in Western Europe and is generally less virulent (5, 6). Originally, *Cryptococcus gattii* was known to be geographically restricted to tropical and subtropical regions (7, 8). However, there has been a surge in *C. gattii* cases beginning in 1999 in the east coast of Vancouver Island, British Columbia that has spread to lower mainland of British Columbia, and the Pacific Northwest of the United States (9, 10). *C. gattii* has been isolated in temperate regions of Europe (Austria, Italy, Spain, and the Netherlands) as well as of Asia (Japan, South Korea) (11, 12).

Human infection is acquired by inhalation of airborne spores or desiccated yeast cells from the environment (13). *Cryptococcus* initially gains access to the respiratory tract. Once inside the lungs, *Cryptococcus* has multiple virulence factors for survival and evasion of host immune responses.

1.2 General background of cryptococcal virulence and host defense

The major virulent factors secreted by *Cryptococcus* include; melanin, capsular polysaccharide, laccase, urease and phospholipase B (14).
1.2.1 Melanin:

Melanin is synthesized through the catalytic activity of laccase confers protection against phagocytosis and oxidative killing by macrophages by acting as an antioxidant, and contributes to extra-pulmonary dissemination (15). In addition, melanin contributes to the cryptococcal cell wall by augmenting its rigidity (16), and also protects *C. neoformans* and other organisms from radioactive damage by absorbing electromagnetic radiations (17).

1.2.2 Capsule:

The cryptococcal capsule is primarily composed of glucuronoxylomannan (GXM), an anionic heteropolymer. Other minor components of the capsule include β-glucans and mannoproteins, which have been found to be highly immunogenic (18, 19). The capsule contributes to virulence by suppressing the host immune response by inhibiting leukocyte migration, and phagocytosis (20, 21). In addition, cryptococcal capsular polysaccharides inhibit cytokine secretion, MHC presentation, as well as inducing FasL expression on macrophages, which causes “by stander” cell death of nearby T-cells (22, 23).

1.2.3 Urease:

The mechanism by which urease causes virulence in *C. neoformans* infection is not fully understood. However, urease has been demonstrated to be essential for traversing epithelial barriers and promotes brain invasion by *C. neoformans* (24, 25). In fact, urease-deficient *C. neoformans* has been shown to be less virulent in intravenous infection of mice, suggesting that urease is an important virulence factor (26).
1.2.4 Phospholipase B:

Phospholipase B (P1b1) secreted by \textit{C. neoformans} enhances fungal survival within phagocytes by degrading membranes of host phagosomes and promoting fungal access to cytoplasmic nutrients (26, 27). In fact, P1b1-deficient \textit{C. neoformans} have decreased invasion of the brain and reduced virulence, underscoring the importance of P1b1 in extrapulmonary dissemination (28). Indeed, P1b1 deletion decreased the frequency of nonlytic exocytosis; a process by which \textit{C. neoformans} extrudes out of phagocytic cells without damaging the fungi or the host cell (29).

1.2.5 Pathogenesis of \textit{Cryptococcus neoformans}

Following the initial acquisition of \textit{C. neoformans}, immune-competent hosts, \textit{Cryptococcus} may produce no symptoms because it is either cleared or controlled by a strong cell-mediated immune response leading to a dormant latent infection (30, 31). In fact serological studies have shown that about 80% of urban dwelling children in New York City have been infected with \textit{C. neoformans}, with no clinical manifestations (32, 33), suggesting that a competent host immune response can control the infection. When host immunity is compromised, the dormant form can be reactivated and disseminated by the hematogenous route to cause meningoencephalitis or systemic infection (34, 35).

Cryptococcal meningitis is the most common fungal infection of the central nervous system and the third most frequent neurological condition in AIDS patients (36). Globally, about 1 million new cases of cryptococcal meningitis occur annually in HIV-infected patients, and over 624,000 die within the first 3 months of diagnosis (2). In fact, in high HIV prevalent regions like
parts of sub-Saharan Africa, cryptococcal meningitis is the leading cause of meningitis; more common than *Neisseria meningitides* and *Streptococcus pneumonia* (37). The mortality is >70% in sub-Saharan Africa, whereas in North America mortality is about 20%. Patients with a suppressed immune system, such as those receiving solid organ transplants or patients with leukemia are also highly susceptible to cryptococcal infection. Transplant patients represent approximately 20-60% of HIV-negative patients with cryptococcosis (38). The ability of *C. gatti* to infect immunocompetent hosts (39, 40), highlights the importance of *Cryptococcus* infection in immunocompromised individuals and the general population. Although there is treatment available, the toxicity of these drugs remain a major problem (41). Importantly, it is worth mentioning that many of the patients suffer relapse after treatment, with more than 50% of relapse occurring in AIDS patients (41).

1.2.6 *Host Immune responses to Cryptococcus infection*

The nasal and upper respiratory airway provide routes for *C. neoformans* entry into the host (42). I will present a general discussion of immunity and host defense to *Cryptococcus* before focusing on NK cells, which are the subject of this thesis.

1.2.6.1 *Responses of macrophages to Cryptococcus:*

Alveolar macrophages are one of the first lines of innate immune defense to *Cryptococcus*. In fact, macrophages bind, internalize and kill *C. neoformans* in the lungs (43). Study of the interactions between macrophages and *C. neoformans* has demonstrated that *C. neoformans* is an intracellular parasite (43). Phagocytized *Cryptoccocus* might be killed by macrophages, but the fungal pathogen has evolved mechanisms to evade this host response by exploiting macrophages (44). After phagocytosis, *C. neoformans* can survive and proliferate.
within infected host macrophages, leading to host cell lysis (44, 45), or expulsion of viable fungal pathogen out of macrophages (46, 47). These observations raise the issue of whether macrophages exert a beneficial or deleterious effect during infection. In fact, recent studies have demonstrated that the absence of macrophages or monocytes correlated with prolonged survival in mice (48, 49). Once the bronchial epithelium is invaded by *C. neoformans*, there is interplay of many immune cells to control and clear the fungus.

1.2.6.2 *The complement system:*

The complement system is one of the major host anticryptococcal mechanisms. In animal models, depletion of complement components (C3 to C9) with cobra venom in mice and guinea pigs led to lower survival time, and aberrant ability to clear *C. neoformans* from extraneural sites (50), suggesting the importance of complement in host anticryptococcal defense. In addition, C5-deficient mice succumb three times more to intravenous *C. neoformans* challenge than C5-competent mice (51). Furthermore, patients with cryptococcal burden have reduced levels of C3 and alternative complement factor B (52). The major functions of the complement system in cryptococcosis are to stimulate chemotaxis of phagocytic cells and also to enhance uptake of *Cryptococcus* by phagocytic cells (53, 54).

1.2.6.3 *Responses of Neutrophils to Cryptococcus:*

Neutrophils are believed to be important in clearing *C. neoformans*, as they are recruited to the site of infection and have been demonstrated to effectively kill *C. neoformans* via the oxidative burst, and defensins (55-57).
1.2.6.4 Responses of Dendritic cells (DCs) to Cryptococcus:

In addition to their ability to directly kill *C. neoformans*, both murine and human dendritic cells phagocytose the fungal pathogen and are the major and most efficient antigen presenting cells (58, 59). Dendritic cells are believed to be the major initiators of cell-mediated immunity to anticytococcal activity (60), and are more efficient at inducing T-cell responses to *C. neoformans* infection than macrophages (58, 61). Indeed, in vivo studies have demonstrated that DC recruitment and maturation are required for protective TH1 immune responses against *C. neoformans* (62).

1.2.6.5 Responses of T-cells to Cryptococcus:

The requirement for T-cell immunity to *C. neoformans* is demonstrated by the drastic increase in cryptococcosis with the onset of the AIDS pandemic (2). Besides HIV patients, patients with defects in T-cell immunity, leukemia and lymphoma have high risk of cryptococcal infections (63, 64). In vivo experiments demonstrated that athymic mice and rats that are T-cell deficient are more susceptible to virulent and avirulent strains of *C. neoformans* (65-67). Furthermore, adoptive transfer of mouse splenocytes or serum demonstrated that, whereas T-cell are important in anticytococcal activity, antibodies did not confer immunity to the naïve host (68, 69). T-helper (Th-1) responses are associated with protection against cryptococcal infection (70). The Th-1 immune response is driven by IL-12 and IFN-γ, and it has been demonstrated that IFN-γ knocked out mice have impaired ability to clear *C. neoformans* (70, 71). In fact, a Th1 environment in mice has been shown to completely protected them from both primary infection and secondary infection with *C. neoformans* (72). On the contrary, Th-2 responses exacerbates *C. neoformans* infection. In fact, important Th-2-associated cytokines; IL-4 and IL-10 knockout
mice resolved *C. neoformans* infection at a higher rate than wildtype mice (73). Indeed, IL-4 and IL-10 treated mice were susceptible to infection with increased fungal presence in the brain (73, 74). These observations demonstrate that Th2 responses unlike Th1 immune responses are non-protective in cryptococcal infection.

T-cells exhibit their anticryptococcal activities directly via cytotoxicity or indirectly by cytokine regulatory functions. Both CD4+ and CD8+ T-cells kill *Cryptococcus* directly, using granulysin as the effector molecule upon activation with IL-2/IL-15 and IL-15, respectively. NK cells on the other hand require no cytokine activation but directly kill *Cryptococcus* using perforin as the effector molecule (75-77). In addition to direct T-cell anticryptococcal immune response, the indirect T-cell regulatory responses play important roles in the complex host anticryptococcal immune response. Th1 cytokines activate other immune cells such as CD8+ T-cells, NK cells, as well as macrophages (78). Indeed, Th-1 associated classical macrophages and DCs form giant cells and granulomas to contain the fungal pathogen during primary immune response to infection in immunocompetent hosts (79, 80).

1.2.6.6 Responses of B-cells to *Cryptococcus*:

Antibody-mediated anticryptococcal immune response has been demonstrated to have both positive and negative effects on host defense. Whereas the presence of anticryptococcal antibodies is associated with enhanced recovery in patients with cryptococcal-associated meningitis, absence of antibodies is associated with poor prognosis (81). In addition, passive transfer of capsule-binding antibodies have been associated with prolong survival and reduced fungal burdens (82, 83). The mechanism of action of these protective antibodies is by opsonizing *Cryptococcus* and enhancing phagocytosis by phagocytic cells (84). Despite the seemingly
importance of antibodies in host anticryptococcal response, several studies have demonstrated that antibodies are deleterious to the host. In fact it has been demonstrated that some antibodies against *C. neoformans* are disease enhancing while others are non-protective (85-87). These contradictory studies highlight the complexity of antibody-mediated immunity in host immune response to *C. neoformans* infection. In addition to cognate T cell immunity, NK cells play essential role in host immune defense to *Cryptococcus* by directly killing the fungi or augmenting other immune cells via cytokine production (88).
1.3.1 Responses of Natural Killer cells (NK) to Cryptococcus:

Natural killer (NK) cells constitute 5 to 20% of peripheral blood mononuclear cells and are usually defined as CD16+ CD56+ CD3- cells (89). There are two subpopulations of NK cells based on the density of CD16 and CD56 expression on their surface. CD16^{bright}/CD56^{dim} expressing NK cells constitute 90%–95% of the total NK cell pool and possess a high cytotoxic potential (90). The other 5-10% are CD16^{dim}/CD56^{bright} expressing NK cells that display little cytotoxicity, and mainly produce cytokines upon activation (90, 91). NK cells are lymphocytes of the innate immune system that kill tumor targets by deploying lytic granules containing perforin, granulysin, and granzymes or by expressing the death receptor (89, 92).

In addition to cytotoxicity against tumor and viral-infected cells, NK cells exhibit direct antifungal activity against various fungal pathogens. Both murine and human NK cells exhibit antifungal activity against *Aspergillus fumigatus, Candida albicans, Paracoccidioides brasiliensis*, and *Rhizopus oryzae* (93-97). Indeed, depletion of NK cells result in a significantly impaired ability of infected mice to kill and clear *A. fumigatus, C. albicans*, and *Histoplasma capsulatum* fungal pathogens (98-100). NK cells from both mice and humans have been shown to directly bind and kill *Cryptococcus neoformans* (next section).

1.3.1.1 In vivo studies of NK cell anticryptococcal activity:

A number of in vivo studies have demonstrated that NK cells are effective in inhibiting cryptococci. An earlier study utilized cyclophosphamide to deplete mice NK cells and other effector cells, and then challenged them with *C. neoformans* (101). Mice treated with cyclophosphamide had higher CFU burden in the spleen, lungs, and liver compared to control mice (101). When untreated nylon wool-nonadherent (NWN) spleen cells which are
predominantly NK cells were adoptively transferred into cyclophosphamide-treated mice, their impaired ability to clear *C. neoformans* was restored. In contrast, depletion of NK cells in the NWN spleen cells with anti-asialo GM1 and complement treatment prior to adoptive transfer of NWN spleen cells did not restore the defective clearance of *C. neoformans* in the spleen, lungs, and liver (101), suggesting that NK cells are required for the clearance of the fungal pathogen. Further, specifically depletion of NK cells has been shown to compromise the host’s ability to clear *C. neoformans*. In this study, it was demonstrated that mice depleted of NK cells with NK-specific anti-NK 1.1 antibody had higher fungal burden in the lungs compared to untreated mice after *C. neoformans* challenge (102). In addition, a study demonstrated that beige (bg/bg) mice that have defective NK cell activity and abnormal polymorphonuclear leukocytes (PMNL) failed to clear *C. neoformans* effectively compared to their heterozygous (bg/+) littermates (103). The study further showed that the abnormality was because of aberrant NK cell activity and not due to PMNL defect, as PMNLs from bg/bg mice inhibited *C. neoformans* growth better than bg/+ mice, and the ability of splenic macrophages from both groups of mice to kill *C. neoformans* was equivalent (103). Another study went further by specifically depleting NK cells in both bg/bg and bg/+ mice with NK-specific depleting antibody (104). Treatment of bg/+ mice with anti-NK-1.1 abrogated their ability to clear *C. neoformans* in the lungs compared to non-treated bg/+ mice (104). An important observation from this study was that anti-NK-1.1-treated bg/+ mice had similar CFU in the lungs compared to bg/bg mice (104), providing further evidence that NK cells rather than macrophages and/or PMNLs are responsible for *C. neoformans* clearance in the lungs of beige mice. Altogether, these observations demonstrate that NK cells play vital roles in the control and clearance of *C. neoformans* in vivo.
1.3.1.2 In vitro studies of NK cell anticryptococcal activity:

The importance of NK cells in host anticryptococcal immune response was initially demonstrated with murine nylon-wool nonadherent (NWN) spleen cells, which are enriched for NK cells. One such study demonstrated that NWN spleen cells had the ability to significantly inhibit the growth of *C. neoformans*, and that this activity was augmented with Poly I:C; an NK stimulating agent (105). Studies of the interactions between NK cells and *C. neoformans* revealed that binding is required for killing of the fungal pathogen (106-108). In one study, it was demonstrated that NK cells binding to *C. neoformans* was a prerequisite for inhibition of the fungal pathogen, and that this process is dependent on Mg2+ but independent of temperature (109), suggesting that NK cell inhibition of the fungal pathogen is contact-dependent.

Investigation of the binding dynamics demonstrated that NK cells associate with *C. neoformans* through many microvilli, which is in contrast to the membrane-membrane contact made between NK cells and tumor cell targets (110). However, the receptor used by NK cells to bind to *C. neoformans* remained elusive until it was recently discovered to be the natural cytotoxic receptor (NCR), NKp30 (111). The processes involved in NK cell killing post-binding to *C. neoformans* requires sarcoma viral oncogene homology (Src) family kinase → phosphatidylinositol 3 kinase (PI3K) → extracellular signal regulated kinase (ERK)1/2 signaling (112), and polarization of the microtubule-organizing center (MTOC) towards the site of contact (106, 113). These observations suggest that, although tumor and microbial killing are different, the mechanism by which NK cells kill *Cryptococcus* may share some similarities with tumor killing, and that some effector functions may probably be similar.
1.4 Anticryptococcal activity of NK cells from HIV-infected patients

Despite the importance of cryptococcal infection in HIV-infected patients, and the importance of NK cells in the clearance of the fungal pathogen, there are limited studies on NK-cell mediated anticryptococcal activity of HIV-infected patients. One study conducted in the pre-ART era demonstrated that NK cells from HIV-infected patients were unable to kill Cryptococcus (114). Considering this study was done prior to ART, it is logical to investigate if this defect still persists in the present day of advanced ART in HIV-infected patients. Further, the mechanism underlying the defective anticryptococcal activity is unknown. Therefore, deciphering the mechanism underlying this defect will provide potential targets for immunotherapy.

1.5 Effector mechanisms of NK cells

The effector mechanisms involved in NK-cell antimicrobial activity is sparsely understood. However, there is extensive knowledge on how NK cells kill tumor cells. NK cells kill tumor cells by either granule-mediated or Fas/FasL mechanisms (115, 116).

1.5.1 The Fas/FasL pathway:

This pathway involves the binding of Fas ligand (FasL) expressed on NK cells to their cognate receptor Fas expressed on tumor targets which consequently induces apoptosis in the target cell (117). Binding of Fas to FasL, leads to the intracellular domain of Fas binding to the Fas-associated DD-containing protein (FADD), which initiates activation of caspase 3, 6 and 7, and mitochondrial damage (118, 119). The mitochondrial damage result in the release of cytochrome-c, which through its activity activates caspase-3. The cumulative effect of caspases-
3, 6, and 7 cleavage of several cellular substrates leads to apoptotic death of the target cells (119). However, whether this pathway is involved in NK cell microbial killing is unknown.

1.5.2 The granule-mediated pathway:

The granule-mediated mechanism, involves directed release of cytolytic granules into tumor targets to induce apoptosis. Perforin; a pore-forming protein is released to the target, and induces lysis of the target or forms a membrane attack complex in the cell membrane of the tumor cell (120). The creation of pores in the cell membrane of the target cell facilitates the entry of granzyme proteins that induce apoptosis in the target cell (121). This pathway of NK cell killing has been demonstrated to be required for NK-cell antifungal activity, although much less is known about the details of this mechanism.

1.5.3 The granule-mediated killing of tumor cells by NK cells:

In tumor cell killing, binding of NK cells to target cell leads to conformational change of Lymphocyte function associated antigen-1 (LFA-1), this signals the activation of F-actin reorganization, and polymerization via phosphorylation of Vav1 by Src family kinases (122, 123). F-actin accumulation ensures firm adhesion to target cells (124). The next stage in this pathway involves dynein motor-dependent movement and convergence of lytic granules on the MTOC (125). Convergence of the granules on the MTOC is an important stage in committing the NK cell to cytotoxicity. However, the movement of the MTOC to the IS (polarization) is independent of granule convergence (126), suggesting that a different mechanism is involved in granule polarization. Following the convergence of the lytic granules onto the MTOC, the
MTOC with the associated lytic granules reorient and polarize toward the NK cell-tumor synapse in an ERK2 phosphorylation-dependent mechanism (127).

The mechanism underlying MTOC polarization is believed to be initiated by the interaction between kinesin-1 with the small GTPase Arl8b (128). F-actin polarization is required for MTOC polarization, and studies have shown that interference with F-actin dynamics impairs MTOC polarization. In one study, knockdown of WASp-interacting protein (WIP), an important protein in actin cytoskeleton rearrangement inhibited polarization of lytic granules to the IS (129). Another study showed that knockdown of phosphorylated forms of the coractin homolog (HS1), which is essential for actin assembly resulted in defective granule polarization to the IS, suggesting that actin polymerization is essential for MTOC movement to the IS (130). The plus ends of microtubules and cortical actin involved in the MTOC movement to the synapse is believed to be linked by IQ motif containing GTPase-activating protein 1 (IQGAP1) (128). Indeed, siRNA silencing of IQGAP1 resulted in failure of the MTOC to polarize to the synapse, and consequently loss of cytotoxicity of YTS (NK cell-line) cells (131). These observations propose a model in which IQGAP1 anchors cortical actin and pulls the MTOC toward the synapse via binding to the plus ends of microtubules. Associated with the MTOC polarization is the reorientation of the Golgi along with microtubules toward the synapse, and this process is believed to aid the directed secretion of lytic granules toward the target cell (132). After polarization, the MTOC together with the lytic granules then dock and fuse with the plasma membrane leading to release of granule contents to kill the target cell (133). The termination of this process involves detachment and vesicle recycling (133). The processes involved in the granule-mediated killing of tumor by NK cells have not been explored in detail in HIV-infected patients. One study demonstrated that NK cells from HIV-infected patients failed
to rearrange tubulin after binding to tumor cells (134), suggesting that there might be a defect in granule mobilization in NK cells from HIV-infected patients. However, whether cytolytic granule polarization to tumor targets is defective in NK cells from HIV-infected patients is unknown.

1.5.4 The granule-mediated killing of Cryptococcus by NK cells:

In direct NK cell anticryptococcal activity, intact actin in the NK-cell cytoskeletal network is required (110), suggesting that actin plays an important role in the NK-cell killing of the fungal pathogen. In fact, NK cells rearrange their cytoskeletal network after binding to C. neoformans, consequently, inhibition of microtubule assembly with colchicine abrogated NK cell killing of the fungal pathogen (106). Further, blocking of Golgi transport with the drug monensin abrogates NK-cell killing of C. neoformans (107), suggesting that movement of lytic granules is important in NK-cell anticryptococcal activity. However, whether granule polarization is required for NK cell killing of Cryptococcus is unknown. Further analysis of the importance of granules to NK-cell anticryptococcal activity demonstrated that, granules isolated from NK cells inhibited C. neoformans growth (107), and that exocytosis of these granules are required for NK cells to kill the fungal pathogen (107), suggesting that granule release is required in NK cell anticryptococcal activity.

1.5.5 Perforin as the effector lytic in NK cell anticryptococcal activity:

Perforin is a pore-forming protein that is constitutively expressed in NK cells (135). Perforin is synthesized as a 555 amino-acid, 65 kD biologically inactive form in the endoplasmic reticulum (ER), where it is transported to the Golgi apparatus. From the Golgi apparatus, it is
eventually transported to the lytic granules (136). In the lytic granules, the biologically inactive perforin is subjected to proteolytic cleavage by cathepsin L through the removal of the last 20 amino-acids at the C-terminus to produce the active form (137). The N-terminus of perforin contains the membrane-attack complex/perforin domain (MACPF) that is responsible for the lytic activity of perforin (120). Following the release of perforin to the target cell in the presence of calcium (Ca\textsuperscript{2+}), perforin inserts itself into the target membrane, creating pores that destabilizes the osmotic balance leading to lysis of tumor cells (138). Alternatively, the pores generated by perforin in the target cell facilitate the entry of granzymes that induce apoptosis in tumor targets (139). Studies have shown that perforin is used by NK cells in antifungal cytotoxicity against fungi such as A. fumigatus, Rhizopus oryzae and C. neoformans (77, 94, 140). In NK-cell cryptococcal killing, inhibition of perforin by concanamycin A, a chemical that causes accelerated degradation of perforin via increase in pH of lytic granules (141) or by small hairpin interfering RNA (shRNA) abrogated NK cell killing of the fungal pathogen (77), demonstrating that perforin is important in NK cell killing process. This is in contrast with CD4+ and CD8+ T-cell killing of Cryptococcus, which require granulysin as the effector molecule (75, 76). Taken together, our current knowledge of the direct NK-cell anticytococcal activity establishes a model that; NK cells use perforin in killing of C. neoformans. However, whether perforin is released independent of polarization (142) or via polarization of lytic granules to the NK-fungal synapse is not known.

1.6 Immune defects in HIV-infected patients

The hallmark of HIV infection is the infection and destroying of cells expressing CD4 on their surface, which are predominantly CD4+ T-cells (143). Indeed, during the period of
asymptomatic infection, both virus-specific memory T cells and naïve T cells are depleted (144). Further, HIV infection induces CD4+ T cell functional defects including defects in antigen recognition (145). In addition to CD4+ T cell destruction, HIV-infection induces immune dysfunction in both host adaptive and innate immune responses (146). CD8+ cytotoxic T lymphocytes from HIV-infected patients fail to co-express perforin with granzyme A within cytotoxic granules (147), suggesting that CTL granule-mediated cytolysis is impaired in HIV-infected patients. Further, it has been demonstrated that CTLs in gut-associated lymphoid tissues do not express perforin (148), suggesting that HIV subverts perforin in mucosal surfaces to prevent lysis of infected cells. Several immune cells including B cells, macrophages, NK cells, and dendritic cells are dysfunctional in HIV-infection (146, 149, 150). The mechanisms underlying HIV dysregulation of host immune system are generally believed to be as a result of HIV-induced increased turnover and differentiation of immune cells (151).

Antiretroviral therapy has revolutionized the natural course of HIV infection. The availability of ART has dramatically increased the life expectancy and quality of life of HIV-infected patients (152). In a retrospective study, it was demonstrated that the mortality of HIV-infected patients declined from 987 to 78, when 3 years of pre-ART deaths were compared with 3 years post-ART (153). Another study demonstrated that ART increased the survival time of AIDS patients as well as HIV-infected non-AIDS patients by 10.6 years and 21.5 years respectively (154). Further, it has been demonstrated that there was correlation between newer ART development and increased survival of HIV-infected patients (155), suggesting that effective ART enhances life expectancy of HIV-infected patients. The success of ART is attributed to its ability to suppress viral replication, which consequently leads to increase in CD4+ T cell count in HIV-infected patients (156). Indeed, ART decreases the rate of turnover in
CD4+ and CD8+ T cells, which results in prolonged survival and increase in T cell count and immune responses (157). Further, it has been demonstrated that ART is able to recover defects in CD4+ T helper cell responses (158). In addition to T cells, ART restores some B cell functions, such as HIV-specific B-cell responses, including hypergammaglobulinaemia (159). The increased turnover of NK cells, B cells, CD4+ and CD8+ T cells are reversed with ART in SIV-infected monkeys (160), suggesting that ART can reverse some immune dysregulation in HIV infection. Despite the ability of ART to reverse some aspects of immune dysfunction in HIV infection, several host immune responses remain defective. In fact, about 20% of HIV-infected patients receiving ART do not respond with an increase in CD4+ T cell count despite virologic suppression (161), suggesting that ART is limited in reversing CD4+ T cell loss in HIV infection. CTL cytolytic activities remain defective during ART (162), and the ability of HIV Env to stimulate perforin mRNA in C8+ T cells remained defective irrespective of ART (163), suggesting that critical cytolytic activities of CTLs remained defective with ART. Further, critical B cell responses such as memory B-cell functions are not recovered with ART (164), suggesting that HIV-infected patients have defective ability to elicit secondary immune responses to foreign antigens. Indeed, it has been demonstrated that in vivo immune responses to immunization is impaired in patients receiving ART (165). In addition to persistence of defects in the adaptive immune responses of HIV-infected patients during ART, innate immune responses also remain defective. Despite ART, NK cell and plasmacytoid dendritic cell activities are incompletely recovered after over a year of active therapy (166), suggesting that critical functions of both adaptive and innate immunities remain defective with ART.
1.7 NK cell defects in HIV-infected patients

In light of the importance of NK cells in microbial host defense, it is particularly tragic that NK cells from HIV-infected patients have impaired functions (167). It is worth mentioning that these studies were focused on NK cell and tumor interactions, with little to no information on NK cell interaction with life-threatening microbes in HIV-infected patients. NK cells from HIV-infected patients have various phenotypic and functional defects, such as high expression of inhibitory natural killer receptors (iNKRs), low levels of natural cytotoxic receptors (NCRs), and reduced cytotoxicity capacity to tumor cells (167-169). The expression of lymphocyte function-associated antigen 1 (LFA-1), an important adhesion molecule required for granule polarization and NK-cell binding to tumor cell was shown to be decreased on NK cells from HIV-infected patients (170). In fact, HIV-gp120 binding to CD4 has been shown to inhibit LFA-1-mediated interaction with tumor cells by dissociating LFA-1 from its cytoplasmic partner (171), further compromising the ability of the NK cells to bind to target cells. Indeed, as HIV-infection progresses in the host, there is a decrease in the more cytotoxic CD16$^{\text{bright}}$/CD56$^{\text{dim}}$ NK cell subset with a consequent expansion of CD16$^{\text{dim}}$/CD56$^{-}$ NK cells that express killer inhibitory receptor (KIR) and are non-cytotoxic (172, 173). Indeed, lower perforin expression has been demonstrated in NK cells and cytotoxic T cells of HIV-infected cells (174) (175, 176). One study demonstrated that perforin mRNA expression was lower in CD8+ T cells in the lymphoid tissues of HIV-infected patients (177). However, whether perforin synthesis is defective in NK cells from HIV-infected patients and whether ART reverses this defect is unknown. Another important observation was reported by a study, which demonstrated that the HIV protein Tat inhibited NK cell-mediated lysis of tumor cells, by blocking L-type Ca$^{++}$ channels which are essential for microtubule remodeling and cytolytic granule degranulation (178). Altogether, these
observations demonstrate that HIV infection leads to compromised cytotoxic capacity of NK cells to tumor cells.

ART partially restores NK cell activity over a long period of treatment, but certain critical functions of NK cells remain compromised (179). It has been demonstrated that ART reversed the expression of iNKR expression on NK cells from HIV-infected patients (167), suggesting that the balance between activating and inhibitory receptors could be tilted in favor of the former, hence promoting NK cell responses in these patients. The expression of the NK cell co-stimulatory receptor, 2B4 (CD244) was normalized after ART (180), and it was further demonstrated that ART probably normalized changes in the host NK cell compartment (181). Over a yearlong ART was shown to enhance NK cell interactions with DCs (166), suggesting that suppression in HIV viral load can restore some NK cell activities.

ART however, fails to restore important NK cell functions. In one study, patients on ART for over 2 years still had persistent reduction in NK cell activating receptors that are required for NK cell-mediated tumor cytotoxicity (167), suggesting that defective NK cell activation is not reversed with ART. Another study demonstrated that ART over a year failed to fully restore NK cell numbers and functions (166), providing further evidence that NK cell dysfunction is still persistent during active therapy. Although NK/DC cell interactions are enhanced with ART, NK cell cytolytic functions remained defective (166), suggesting that checkpoints in the NK cell cytolytic pathway remained defective with ART. These observations notwithstanding, questions surrounding the critical functional consequences of these persistent defects remain unanswered. Whether NK cells from HIV-infected patients have defects in cytolytic granule mobilization and transport to target cells, and whether ART reverses this defect remained unknown. Also whether NK cells have defective binding to target cells and whether ART reversed this defect is
unknown, suggesting that studies undertaken to answer these questions will not only expand our knowledge in NK cell function in HIV infections but also unravel possible therapeutic targets.

1.7.1 Altered cytokine profile in HIV-infection:

HIV-accessory proteins have been demonstrated to dysregulate the production of several cytokines that are important in NK cell function and Th-1 responses. Indeed, in HIV infection, there is a shift from stimulatory Th1 cytokine response to inhibitory Th2 cytokine responses (182). Further it has been shown that Th2 cytokines are associated with ineffective clearance of HIV-infected cells, suggesting that HIV skews host cytokine responses to facilitate its survival (183). One study demonstrated that the gp120 protein of HIV induces IL-10, IFN-β, and TNF-α production (184), suggesting that HIV has the ability to dysregulate the cytokine profile of the host.

1.8 Important cytokines in NK cell-mediated anticytotoxic response

Several cytokines including IL-2, IL-6, IL-12, IL-15, IL-21, IFN-α/β and IFN-γ promote NK cell survival and cytotoxicity to tumor cells (185-187). Both IL-2 and IL-15 promote proliferation, differentiation and survival of NK cells (188). In NK-cell cytotoxic, IL-2 has been demonstrated to be a potent up regulator of perforin in NK cells, whereas IL-15 enhances NK cell cytotoxicity by upregulating NKG2D activating receptors on NK cells (189, 190). However, in the complex host anticytotoxic immune response, IFN-γ and especially IL-12 have been demonstrated as the main drivers of protection (191, 192).
1.9 Interleukin-12 (IL-12) regulation of NK cell effector activities:

IL-12 is a pleiotropic heterodimeric cytokine composed of two disulfide-linked subunits designated p35 and p40 that form the bioactive IL-12p70 (193). The genes encoding the p40 and p35 have been mapped to the human chromosomes 5q31-q33 and 3p12-3q13.2 respectively (194). The main source of IL-12 in humans is from dendritic cells, and to a lesser extent other phagocytic cells such as neutrophils, monocytes and macrophages (195, 196). During host immune response to infections, there is increase in the bioactive IL-12-p70 heterodimer via priming and amplification processes (197). Priming is mediated via toll-like receptor (TLR) family. It has been shown that binding of TLR4 to lipopolysaccharide (LPS) and binding of TLR7/8 to R848 induce IL-12 production by macrophages (198), suggesting the requirement of these receptors in the priming process. The amplification process of IL-12 production can be achieved by one of two mechanisms. The first mechanism involves direct cell-cell contact of the producer cell with other immune cells. Indeed, it has been shown that direct contact between T–cells and dendritic cells via CD40L-CD40 ligation enhanced IL-12 production (199). The other mechanism of IL-12 amplification involves the activity of other cytokines. Treatment of dendritic cells (DC) with IL-1β has been shown to induce IL-12 production in a dose dependent manner (200), suggesting the regulatory role of IL-1β in IL-12 production.

Binding of the IL-12 heterodimer to the IL-12 receptor activates the Jak kinases (Tyk-2 and Jak-2), leading to signaling through STAT-4 (201), which consequently activates several biological activities. The most profound effect of IL-12 on NK cells is its potent stimulus of NK cell cytotoxicity to tumor cells. Indeed several studies have documented the potent effect of IL-12 on both murine and human NK cell cytotoxicity. In one study, it was shown that IL-12 enhanced NK cell cytotoxicity against tumor cells via upregulating NKG2D, an activating
receptor of NK cells (202). Another study demonstrated that NK cell cytotoxicity to Jukart cells was dependent on IL-12-stimulated up regulation of TRAIL activating receptor on NK cells (203). In addition, signals that are crucial in NK cell cytotoxicity are promoted with IL-12 treatment, as demonstrated by phosphorylation of Erk1/2 in NK cells (202). Other studies have shown that IL-12 enhances NK cell antitumor activity by potentiating the perforin-mediated apoptosis and cytolytic pathway (204, 205). However, it is unknown whether IL-12 potentiates NK cell cytolytic pathway by promoting perforin polarization and release to tumor targets. IL-12 was initially designated as “cytotoxic lymphocyte maturation factor” due to its potent effect on NK and T cell cytotoxicity (206, 207). Further, IL-12 stimulates NK cells to produce IFN-γ, which is the main cytokine responsible for inducing Th1 phenotype (208), that confers protection in Cryptococcus infection.

1.9.1 Interleukin-12 (IL-12) and the protection against C. neoformans

The importance of IL-12 in host antifungal immunity is well documented. One study demonstrated that the susceptibility of mice to C. neoformans was enhanced in the absence of IL-12p35 or IL-12p40 (191). Another study demonstrated that IL-12 administration to infected mice significantly reduced fungal burden in the lungs and prevented cryptococcal meningitis compared to untreated control mice (209). Indeed IL-12-knockout mice have also been demonstrated to be susceptible to Blastomyces and Candida fungal pathogens (210, 211). Further, it has been shown that administration of recombinant IL-12 promoted clearance of Histoplasma capsulatum and Candida in IL-12p40^{-} mice (211, 212). Importantly, patients with congenital disorders in IL-12 receptors have been shown to be more susceptible to Paracoccidioides and Coccidioides fungal infections (213, 214), underscoring the importance of
IL-12 in host antifungal immunity. IL-12 promotes NK cell-mediated antifungal activity in two ways; (1). By promoting NK cell mediated direct antifungal activity or (2) by promoting NK-cell mediated Th-1 response through IFN-γ production. In addition to its effect on NK cell-mediated antifungal immunity, IL-12 is known to enhance host immunity by promoting T helper cell development and activation of macrophages (215, 216).

1.9.2 Interleukin-12 (IL-12) and direct NK-cell anticryptococcal activity

There is a gap in our understanding of the role of IL-12 in NK-cell direct antifungal activity. The only direct evidence demonstrated that in vitro treatment of purified NK cells from HIV-infected patients with IL-12 restored their defective anticryptococcal activity (114). One study demonstrated that HIV Tat protein inhibited IL-12 production and induced the expression of IL-6 and TGF-β1, which resulted in inhibition of NK cell-mediated Th-1 response (217). Importantly, HIV-infected patients have low IL-12 production. The mechanism involves the suppression of IL-12 p35 production resulting in defective release of the biologically active IL-12 heterodimer in monocytic lineages by HIV-vpr (218). In effect, this aberrant IL-12 response during HIV-infection dysregulates NK cell responses to both tumor and fungal targets.

Considering the perforin-mediated cytotoxic pathway is essential for direct NK cell anticryptococcal activity, and that this pathway is enhanced with IL-12 treatment in NK cell antitumor activity. It will be interesting and logical to investigate the mechanism by which IL-12 restores NK cell-anticryptococcal activity of HIV-infected patients.
1.9.3 Interleukin-12 (IL-12) and indirect NK cell-mediated anticryptococcal activity

The role of IL-12 in indirect NK-cell mediated Th-1 response to fungal infections is well studied. The mechanism involves the secretion of IFN-γ, which is the key driver of a Th-1 response. In fact, one study demonstrated that IL-12-deficient mice had impaired IFN-γ production that consequently led to impaired Th-1 response (219). Another study demonstrated that IL-12p35\(^{-/-}\) and IL-12p40\(^{-/-}\) mice had deficient IFN-γ production resulting in aberrant Th-1 response, and were not protected after *C. neoformans* challenge (191), highlighting the sequential link between IL-12 and Th-1 response. Further, it was demonstrated that administration of IL-12 to mice induced IFN-γ production that conferred protection against *C. neoformans* infection (209). In *Aspergillus fumigatus* infection in mice, IL-12-engineered DCs that overexpressed IL-12 were demonstrated to confer protection through a robust *Aspergillus*-specific IFN-γ response (220), suggesting the ability of IL-12 to protect against the fungal pathogen. Further investigations into the underlying mechanism of IL-12-induced protection showed that, IL-12 induced IFN-γ production by NK cells, IFN-γ together with IL-12 induced T-bet expression (221). T-bet suppresses Th-17 development, and consequently skews the immune response to a Th-1 phenotype (222). Altogether, these observations demonstrate that whereas IL-12-mediated indirect NK cell anticryptococcal activity is well studied, there is not much known about IL-12-mediated direct anticryptococcal activity. Hence investigating the effect of IL-12 on the direct NK cell anticryptococcal activity provides opportunity to unravel mechanisms that might be of therapeutic importance, as well as adding to the current knowledge in the field.
1.9.4 Interleukin-12 (IL-12) as an adjunct therapy in HIV-infected patients

IL-12 has been administered to both HIV-infected patients and SIV-infected monkeys. IL-12 therapy has been effectively administered to patients with Kaposi’s sarcoma, for which other therapies have failed (223, 224), suggesting its importance in the treatment of opportunistic infections. Further, IL-12 administered in the absence of antiretroviral therapy to Indian Rhesus macaques infected with simian immunodeficiency virus led to a marked increase in the frequency of circulating NK cells and restoration of NK lytic functions (225, 226). Using IL-12 as an adjuvant therapeutic agent was discouraged due to its high toxicity to the host (223). However, recent studies employing different therapeutic approaches have shown less toxicity and success in treating leukemia (227, 228). Considering that IL-12 can restore defective NK-cell antifungal activity of HIV-infected patients, coupled with its usage in successfully treated Kaposi’s sarcoma, suggest that there is potential for cytokines such as IL-12 in antimicrobial immunotherapy for HIV-infected patients.
**Hypothesis:** I hypothesize that NK cells from HIV-infected patients have multiple defects in direct anticryptococcal activity that are restored by IL-12-treatment.

**Aim 1:** To determine whether NK cells from HIV-infected patients have defective anticryptococcal killing in the post-ART era

**Aim 2:** To determine the mechanism underlying the defective direct NK cell anticryptococcal activity of HIV-infected patients

**Aim 3:** To determine the role of IL-12 in direct NK cell anticryptococcal activity of HIV-infected patients
CHAPTER TWO: MATERIALS AND METHODS

2.1 Study population

The primary group studied were HIV-infected adult patients between the ages of 24-55 years receiving ART with CD4+ counts between 300-700 cells/µl with no co-infection. Patients were recruited from the Southern Alberta Clinic, Calgary AB, Canada. Healthy adults between the ages of 20-50 years were recruited from the University of Calgary, Foothills campus. The use of human material was approved by the Conjoint Health Research Ethic Board of the University of Calgary, Calgary, AB, Canada. In cryptococcal killing experiments, HIV-infected patient’s naïve to ART with CD4+ count 300-600cells/µl were recruited. All the patients with the exception of ART naïve patients had no detectable viral load (<69 copies/ml) based on the standard clinical protocol for assessing viral load (Table 1)
Table 1. Laboratory Characteristics of HIV-infected Patients

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2.2 Antibodies

Mouse anti-human CD3-phytoerythrin (PE), CD16-(PE), CD19-(PE), CD56-fluorescein isothiocyanate (FITC), perforin (clone clone δ G9)-(FITC), CD107a-FITC, mouse IgG1-PE, IgG2a-FITC, CD11a-PECy5, and mouse IgG1-PECy5 were purchased from BD Biosciences (Mississauga, Canada). Rabbit polyclonal anti-NKp30 blocking antibody, and rabbit IgG were purchased from Abnova (Abnova, ab27472, Taiwan).

2.3 Cell culture

2.3.1 Primary human NK cells

NK cells from HIV-infected patients and healthy donors were isolated from peripheral blood by negative selection using the RosetteSep Human NK Cell Enrichment Cocktail (STEMCELL, 15065, Vancouver Canada) as per the manufacturer’s instructions. In brief, RosetteSep Human NK Cell Enrichment Cocktail was added at 50µl/ml of whole blood, mixed well and incubated at room temperature for 20 minutes. The sample was then diluted with an equal volume of Phosphate-buffered Saline (PBS) supplemented with 2% Fetal Bovine Serum (FBS), and gently mixed together. The sample was then layered on top of the density gradient medium Ficoll-Paque (GE Healthcare, Quebec, Canada) and centrifuged at 1200xg with brake off for 20 minutes at room temperature. The enriched cells were removed from the density medium: plasma interface, and washed twice with PBS+2% FBS.
2.3.2 *Cryptococcus neoformans*

*C. neoformans* strain B3501 acquired from the American Type Culture Collection (ATCC 34873, Manassas, VA. USA) was maintained and cultured in Sabouraud dextrose broth and agar (Becton Dickinson [BD]). For use in experiments, *C. neoformans* was grown to the log phase in Sabouraud dextrose broth at 32°C with gentle shaking. The organisms were vigorously vortexed to reduce clumping, and diluted in trypan blue before counting in a hemocytometer chamber prior to use in experiments.

2.3.3 Anticryptococcal activity

Anticryptococcal activity was assessed by the determination of the number of colony forming units (CFU) as previously described (77, 111). Briefly, NK cells (1x10⁵) were pretreated with or without 100IU of rh-IL-12 (R&D systems) for 20hrs, and co-cultured with *C. neoformans* (1x10³) in identical quadruplicate wells of 96 well plate at 37°C for 24 hours. In experiments demonstrating granule-dependent killing of *C. neoformans*, IL-12 treated NK cells were treated with or without 10µm strontium chloride SrCl₂ for 24 hrs (Sigma-Aldrich) as described previously (93). The CFU were determined at 0 hrs (starting inoculum) by plating the contents of the quadruplicate wells containing *C. neoformans* alone onto Sabouraud dextrose agar plates. After 24 hours of incubation, serial dilutions of control and experimental quadruplicate wells were performed and platted onto agar plates for CFU determination.

2.4 Degranulation assay

NK cell degranulation following stimulation with *C. neoformans* was assessed by a flow cytometry as previously described (229) with modifications. In brief, NK cells were labelled at 37°C in 5% CO₂ for 20 minutes with PECy5-conjugated mouse anti-CD11a antibody (BD Biosciences) at 5µl/1x10⁵ NK cells in Fluorescence-activated cell sorter (FACS) buffer and
washed three times with PBS. Anti-CD11a-PECy5-labelled NK cells (1x10^5) were cultured with *C. neoformans* in the presence of FITC-conjugated anti-CD107a monoclonal antibody (BD Biosciences, clone H4A3). The samples were incubated in 5% CO_2 at 37°C for different length of times (30mins, 180mins, 240mins). The reaction was terminated with 3% formalin at the end of the incubation period. Samples were washed three times with PBS, and the percentage of CD107a positive cells were measured by flow cytometry with a Guava EasyCyte flow cytometer (Guava Technologies, San Fransisco, CA). The data were analyzed with the FlowJo software package (Tree Star, Ashland, OR, USA).

### 2.5 Determination of stimulated perforin released

NK cells (1x10^5) were treated with or without rhIL-12 for 18hrs, then cultured with or without *C. neoformans*. The cells were centrifuged at 800xg and the supernatants were collected. Perforin released into the culture media was assessed by ELISA (Abcam, ab46115, Canada), following the manufacturer’s instructions. The optical density was measured using a SpectraMax M2e Multi-Mode Microplate Reader (Molecular Devices, CA. USA). To determine perforin release, NK cells were washed with fresh media prior to stimulation with *C. neoformans* for different length of times (0.5hr, 1hr, 2hrs). The supernatants were collected and analysed for perforin. “Stimulated perforin release” was calculated by subtracting the amount of perforin in supernatants of unstimulated cells from the amount of perforin in supernatant from cells stimulated with *Cryptococcus*.

### 2.6 Intracellular perforin content

NK cells were treated with or without rhIL-12 for 18hrs. The cells were then fixed and made permeable using Cytofix/Cytoperm (BD Biosciences) according to the manufacturer’s instructions. NK cells were then labeled with 5µl working concentration of FITC-conjugated
anti-perforin antibody per 100µl experimental sample (BD Biosciences, 556577, clone δ G9) in FACS buffer (PBS, 1% Bovine serum albumin (BSA), 10% Fetal Bovine Serum (FBS), 0.1% NaN₃ sodium azide), or isotype-matched control antibody (BD Biosciences). The sample was washed twice with PBS, and then suspended in 200µl of PBS for flow cytometric analysis. The fluorescent intensity was measured by flow cytometry with a Guava EasyCyte flow cytometer (Guava Technologies, San Francisco, CA). The data were analyzed with the FlowJo software package (Tree Star, Ashland, OR, USA).

2.7 Sample preparation and Optical Microscopy

For immunofluorescence labelling, NK cells (1x10⁶/ml) were cultured with C. neoformans (2x10⁵/ml) for 1 hour, fixed with 3.5% paraformaldehyde for 30 minutes. The cells were washed twice with 1x PBS, and permeabilized with 1x Perm/Wash (BD Biosciences, 51-2091KZ) for 45 minutes at room temperature. NK cells were then labeled with 5µl working concentration of FITC-conjugated anti-perforin antibody per 100µl experimental sample (BD Biosciences, 556577, clone δ G9) in FACS buffer (PBS, 1% BSA, 10% FBS, 0.1% NaN3 sodium azide), 0.1µg/ml of DAPI (Calbiochem 268298, ON Canada) for nucleus, and Alexa Fluor-647-conjugated phalloidin for F-actin (Invitrogen A22284, ON. Canada). The samples were transferred onto cover slips (Fisher scientific 12-542-B, ON. Canada), air-dried for 30 minutes and mounted with Prolong Gold mounting medium (Invitrogen, P36930). The images were captured with the Volocity acquisition software version 6.2.3 (PerkinElmer, MA, U.S.A) on the wide-field fluorescence microscope (Olympus IX70, ON Canada), using a PlanoApo 60x/1.40 N.A. objective. Matched control and test samples were prepared on the same day, and
images of the same number of contacts of both sets were also acquired on the same day with identical microscope settings.

2.8 Image Analysis

NK cells that were bound to *C. neoformans* as seen by phase contrast were selected for all conditions. The center of the point of contact of NK cells to *C. neoformans* was chosen manually and used as the reference point. The center of the point of contact was defined as the midpoint of the contact interface between an NK cell and *C. neoformans* (Intro. Figure 1). The same threshold of intensity was manually set with the Volocity software and applied to both control and test samples and the areas of fluorescence that were smaller than 0.05µm in diameter were excluded, as they were felt no to represent granules. The same threshold settings of fluorescence intensity and size of granules were used in analyzing both the control and test samples on the same day. The distance of perforin-labelled granules to *C. neoformans* was assessed by measuring the distance from the centroid of each granule in an NK cell to the center of the point of contact with *C. neoformans*. The average measurement represents the average, non-weighted measurement of the distances. The mean of the distances generated per cell was calculated, which represents one data point using the Volocity image analysis software version 6.2.3 (PerkinElmer, U.S.A). The data were exported to Graphpad Prism and graphed. Contrast and brightness of the images were enhanced for visualization purpose only, with no distortion, elimination, or obscuring of any structure in the original image using the Volocity image analysis software version 6.2.3.
Introductory Figure 1: measuring of perforin containing granules to the NK cell-cryptococcal synapse.

The midpoint of the contact interface between an NK cell and *C. neoformans* was determined manually. This was used as the reference point for the automatic measurement of the distances from the centroid of perforin-containing granules to the NK-cryptococcal synapse.
2.9 Binding and conjugate formation

Assessment of conjugate formation of NK cells with *Cryptococcus neoformans* was done as described previously (230). Briefly, NK cells were labelled at 37°C in 5% CO₂ for 20 minutes with PECy5-conjugated mouse anti-CD11a antibody (BD Biosciences) at 5µl/1x10⁵ NK cells in FACS buffer, or with matching isotype control. The NK cells were washed twice with PBS. For *C. neoformans* labelling, 0.1 µM/ml FITC (Sigma, 3326-32-7) was added to 1x10⁶ organisms, and incubated in the absence of light at room temperature for 15 minutes. The sample was washed three times with PBS. NK cells were mixed with *C. neoformans* and incubated at different length of times (5mins, 30mins, 60mins, 120mins). The reaction was terminated with 3% formalin at the end of the incubation period. The sample was thoroughly mixed and conjugates between NK cells and *C. neoformans* were determined by flow cytometry. In NKp30 receptor blocking experiments, the PECy5-CD11a labelled NK cells were cocultured in the presence of rabbit polyclonal anti-NKp30 blocking antibody or rabbit IgG (Abnova, ab27472, Taiwan) for 30 minutes. Anti-NKp30-treated NK cells were cocultured with FITC-labelled *C. neoformans* for 60 minutes and conjugates were determined by flow cytometry analysis.

2.10 Statistical Analyses

Statistical analyses were performed using GraphPad Prism v6.0. Unless otherwise specified, one-way ANOVA followed by Bonferroni comparison tests was used for data with normal distribution or unpaired t tests (two-tailed) with Welch correction were used to determine differences among conditions. Statistical significance was achieved if p< 0.05.
3.1 NK cells from HIV-infected patients have aberrant anticryptococcal activity

NK cells have been shown to directly kill *C. neoformans* in a contact-dependent perforin-mediated mechanism (93, 105, 109). These data were confirmed with freshly isolated and purified human NK cells. Primary NK cells were labelled with anti-human CD3, CD16, CD19 (negative control) and CD56, and analyzed by flow cytometry, which demonstrated approximately 94% purity after isolation (Fig 1.1A). A previous study demonstrated that NK cells from HIV-infected patients with no access to ART had defective anticryptococcal activity (114). To determine whether the defective NK cell anticryptococcal activity of HIV-infected patients extends to the current era of HIV treatment, we queried whether NK cells from HIV patients receiving ART with undetectable plasma RNA viral load (<40 copies/ml) have impaired anti-cryptococcal activity. We co-cultured freshly isolated NK cells with *C. neoformans* and assessed cryptococcal CFU. Cells from healthy donors showed significant anticryptococcal activity at both 24hr and 48 hr (Figure 1.1 B&C). Contrary, there was no significant anticryptococcal activity of NK cells from either ART naïve patients or patients receiving ART (Figure 1.1 B&C). These results confirm that defective NK cell-anticryptococcal activity persist in the current era of HIV treatment.
Figure 1.1: Anticryptococcal activity of NK cells from healthy and HIV-infected subjects

A) NK cells were negatively selected from whole blood by rosette separation using a Ficoll-Hypaque density gradient, labelled with anti-human CD3, CD16, CD19-PE and CD56-FITC and analyzed by flow cytometry. Data is representative of 5 experiments B) NK cells from healthy donors, HIV patients receiving ART (+ART) or no ART (-ART) were incubated with *C. neoformans* (Cn) for 24hr at E: T ratio 100:1. CFU were determined at 0hrs (T0) (starting inoculum) and 24 hrs (T24). Data shown is n= 7 subjects for both healthy and patients on ART (+ART), and n=4 subjects for patients not on ART (-ART). C) NK cells from healthy donors, HIV-infected patients receiving ART (+ART) or no ART (-ART) were incubated with *C. neoformans* for 48hr at E: T ratio 100:1. CFU were determined at 0hrs (T0) (starting inoculum) and 48 hrs (T48). Data shown is n= 7 subjects for both healthy and HIV-infected patients receiving ART (+ART), and n= 4 subjects for patients not on ART (+ART). One way ANOVA with Bonferroni correction was used for statistical significance. Data is the mean ± SEM ** (p< 0.01), *** (p< 0.001), ns (not significant).
3.2 NK cells from HIV-infected patients have defective perforin release in response to *C. neoformans*

Perforin has been identified as the effector molecule employed by human NK cells to Kill *C. neoformans* (77, 207). The observation that NK cells from healthy subjects kill *C. neoformans* but NK cells from HIV-infected subjects do not kill *C. neoformans* (Figure 1.1), suggests that NK cells from HIV-infected subjects might have a defect in their ability to degranulate and secrete perforin. To test this hypothesis, NK cells from both healthy and HIV-infected subjects were challenged with *C. neoformans* at different lengths of time, in the presence of anti-CD107a (LAMP-1) which is a marker of NK cell degranulation (229). The amount of degranulation as demonstrated by the uptake of anti-CD107a was quantified by flow cytometry. NK cells from healthy subjects showed a trend of higher fluorescence intensity of anti-CD107a compared to HIV-infected subjects (Figure 1.2A) suggesting that NK cells from HIV-infected patients had defect in the degranulation process. Further, to specifically determine if perforin release was defective, NK cells from both healthy and HIV-infected subjects were challenged with *C. neoformans* at different lengths of time and the amount of stimulated perforin released into the supernatant was detected by ELISA. NK cells from HIV-infected patients showed lower stimulated perforin release in response to *C. neoformans* compared to healthy subjects (Figure 1.2B). This observation demonstrate that NK cells from HIV-infected patients have defective degranulation and defective perforin secretion in response to *C. neoformans*. 
A

- **HEALTHY**
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B

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- **HEALTHY**
- **HIV**
Figure 1.2: NK cells from HIV-infected subjects had defective perforin release in response to *C. neoformans*.

A) NK cells from healthy donors, HIV patients receiving ART were incubated with *C. neoformans* in the presence of anti-CD107a for different lengths of time. The amount of anti-CD107a taken up by the cells as measured by the fluorescence intensity was assessed by flow cytometry. Data shown is 2 different healthy and 2 different HIV-infected subjects. B) NK cells from both healthy and HIV-infected subjects were challenged with *C. neoformans* at different lengths of time and the amount of stimulated perforin released into the culture media was detected by ELISA. Unpaired t tests (two-tailed) with Welch correction was used to determine statistical significance. Data shown is n= 3 for both healthy and HIV-infected patients receiving ART. Data is the mean ± SEM ** (p< 0.01).
3.3 NK cells from HIV-infected patients have lower intracellular perforin expression

Having demonstrated that NK cells from HIV-infected patients failed to release perforin in response to *C. neoformans*, I considered two possible reasons for this observation. The first possibility may be that NK cells from HIV-infected patients have defective perforin stores. Alternatively, the mechanisms leading to polarization and degranulation might be defective. To investigate if NK cells from HIV-infected patients had a lower expression of perforin required for NK anticryptococcal activity, freshly isolated NK cells were made permeable and labelled with FITC-conjugated anti-perforin antibody that recognizes the active form of perforin that is responsible for cytotoxicity and analyzed by flow cytometry. NK cells from HIV patients showed a lower level of fluorescence compared with NK cells from healthy donors (Figure 1.3A-C), suggesting that NK cells from HIV-infected patients had lower perforin stores. These observations suggest that the defective perforin expression in NK cells from HIV-infected patients may account for the defective NK killing of *Cryptococcus*. 
Figure 1.3: NK cells from HIV-infected patients had lower perforin expression

A). NK cells from healthy donors and HIV-infected patients were freshly isolated, made permeable, labelled with anti-perforin antibody or matching isotype control, and analyzed by flow cytometry. Data is representative of 4 experiments. B). Mean fluorescent intensity of perforin labelling in NK cells from healthy donors and HIV-infected patients (n=3 subjects). Unpaired t tests (two-tailed) with Welch correction was used to determine statistical significance. Data is the mean ± SEM. **p< 0.01.
3.4 NK cells from HIV-infected patients failed to polarize perforin to the synapse with *C. neoformans*.

The results so far show that NK cells from HIV-infected patients have lower perforin stores and defective perforin release. However, for NK cells to deploy their granules, the cytolytic granules are mobilized and polarized to the target (231). Impairment in polarization prevents the perforin molecules from moving toward the microbial synapse prior to release. To investigate if perforin polarization is required in NK cell anticytotoxic activity and whether NK cells from HIV-infected patients are defective in their ability to polarize perforin-containing granules to *C. neoformans*, NK cells from healthy donors and HIV-infected patients were co-cultured respectively with *C. neoformans*. Cells were fixed and intracellular perforin was labelled with FITC-conjugated anti-perforin antibody, and the proximity of the perforin-containing granules to the attached fungi was determined by fluorescence microscopy and image analysis. The distance of perforin to the point of contact with *C. neoformans* reflects the extent to which perforin is polarized. Images demonstrated that perforin-containing granules were more remote from the area of contact with *Cryptococcus* in NK cells from HIV-infected patients (Figure 1.4A), compared to NK cells from healthy donors (Figure 1.4B). Quantitative analysis showed that perforin-containing granules were a greater distance from the area of contact with *C. neoformans* in NK cells from HIV-infected patients compared to NK cells from healthy donors (Figure 1.4C). The increased distance of perforin to the point of contact with *Cryptococcus* suggests that the process leading to polarization was impaired in HIV-infected patients. In addition to lower levels of perforin within the NK cells, defective polarization provided a second mechanism by which perforin release was reduced, leading to defective killing.
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**HEALTHY**

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C  

**Distance of Perforin to C. neoformans (µm)**

![Graph](image13.png)
Figure 1.4: NK cells from HIV-infected patients were unable to polarize perforin to C. neoformans.

NK cells from healthy and HIV-infected patients were challenged with C. neoformans at an E: T ratio of 1:2 for 1hr. The NK cells were labeled with anti-perforin Ab (green), DAPI for nucleus (blue), and phalloidin for F-actin (red) A). Localization of perforin in NK cells from healthy donors bound to C. neoformans at 60X magnification. The traced outline corresponds to the perimeter of the C. neoformans from the phase contrast image and overlaid on the fluorescence image in all panels B). Localization of perforin in NK cells from HIV patients bound to C. neoformans at 60X magnification. The traced outline corresponds to the perimeter of the C. neoformans from the phase contrast image and overlaid on the fluorescence image in all panels. C). The distance of perforin to C. neoformans in NK cells from healthy donors and HIV patients was assessed using Volocity. Each data point is the mean distance of 15 to 30 granules per cell. Data shown is the mean of N=18 cells that had formed conjugates for healthy and N=17 cells that had formed conjugates for HIV-infected patients from 1 healthy donor and 1 HIV-infected patient. The experiment was repeated with different subjects with similar results.

Unpaired t tests (two-tailed) with Welch correction was used to determine statistical significance. **p< 0.01. Data is the mean ± SEM
3.5 NK cells from HIV patients were defective in binding to *C. neoformans*

Contact is a requirement for human NK cell direct killing of *C. neoformans* (77). Having studied the events downstream of the initial contact between NK cells and *C. neoformans*, I sought to determine if NK cells from HIV-infected patients bind with *C. neoformans*. To determine whether NK cells from HIV-infected patients bind to *C. neoformans*, NK cells from healthy donors and HIV patients labelled with PECy5-conjugated CD11a were co-cultured with FITC-labeled *C. neoformans*, for different lengths of time. The cells were incubated together and then passed through the flow cytometry flow cell for analysis. Conjugates were identified when a cell doublet consisting of both red and green fluorescence were detected in the same event by flow cytometry. The percentage of NK cells from HIV-infected patients that formed conjugates with *C. neoformans* was lower when compared to NK cells from healthy subjects (Figure 1.5A). There was a statistically significant higher binding of NK cells from healthy subjects at different lengths of time compared to NK cells from HIV-infected subjects (Figure 1.5B).
A

Healthy

HIV

CD 11a-PEcy5
(NK-Cells)

C. neoformans - FITC

B

HEALTHY

HIV

C. neoformans Binding (%)

Time

5 30 60 120
0 10 20 30

**
**Figure 1.5: NK cells from HIV-infected patients bound less to *C. neoformans*.**

A). CD11a labelled NK cells from healthy donors and HIV-infected patients were cocultured with FITC-labeled *C. neoformans*, for different lengths of time. Conjugates were detected when both red (NK cells) and green fluorescence (*Cryptococcus*) were detected in the same event by flow cytometry. Data is representative of 3 experiments. B). CD11a labelled NK cells from healthy donors (n=4) and HIV-infected subjects (n=4) were co-cultured with FITC-labeled *C. neoformans*, for different lengths of time. Conjugates were determined and quantified. Unpaired t tests (two-tailed) with Welch correction was used to determine statistical significance **p< 0.01. Data is the mean ± SEM.**
3.6 Chapter Summary

In summary, this chapter establishes that NK cells from HIV-infected patients receiving ART have aberrant direct anticytotoxic activity as a consequence of defects in the NK cell cytolytic pathway. It demonstrates that NK cells from HIV-infected patients have lower perforin stores, trend of defective ability to degranulate and defective release perforin in response to C. neoformans challenge, and defective ability to polarize perforin to the NK cell-fungal synapse. Additionally, it demonstrates that NK cells from HIV-infected patients bound less to fungal targets compared to healthy subjects. Thus, there are multiple defects in the cytolytic machinery of NK cells from HIV patients, which cumulatively result in the defective anticytotoxic activity in NK cells from HIV-infected patients.
CHAPTER FOUR: ESTABLISHING THE ROLE OF IL-12 IN NK CELL ANTICRYPTOCOCCAL ACTIVITY OF HIV-INFECTED PATIENTS.

4.1 IL-12 restores antifungal activity of NK cells from HIV-infected patients

Previously, it was demonstrated that aberrant NK cell-anticryptococcal activity of ART naïve HIV-infected patients could be restored by IL-12 treatment (114). However, it was not known whether IL-12 was able to reverse this defect in patients receiving ART. Additionally, the mechanism by which IL-12 restored the ability of NK cells to kill *C. neoformans* was unknown. To determine if IL-12 restores this defect in the current era of HIV treatment, we treated NK cells from healthy donors, HIV-infected patients receiving ART, and ART naïve HIV-infected patients with recombinant human IL-12 for 20 hrs and assessed cryptococcal CFU. NK cells from healthy donors showed no enhanced anticytotoxic activity with IL-12 treatment after 24 hr and 48 hr incubation with *C. neoformans* (Figure 1.2A&B). In contrast, NK cells from both HIV-infected patients receiving ART or no ART showed a significant increase in their anticytotoxic activity (Figures 1.2A&B). These observations indicate that defective anticytotoxic activity of NK cells from HIV-infected patients can be reversed with IL-12-treatment irrespective of ART status.
24 hour killing

A

48 hour killing

B
Figure 2.1: IL-12-treatment restored the defective anticryptococcal activity of HIV-infected patients

A) NK cells from healthy donors, HIV-infected patients receiving ART (+ART) or no ART (-ART) were pretreated with 100IU of rh-IL-12 for 20hrs, and then incubated with C. neoformans (Cn) for 24hrs at E: T ratio 100:1. CFU were determined at 0hrs (T0) (starting inoculum) and 24hrs (T24). Data shown n= 7 subjects for both healthy and patients on ART (+ART), and n=4 subjects for patients not on ART (-ART). B) NK cells from healthy donors, HIV-infected patients receiving ART (+ART) or no ART (-ART), were pretreated with 100IU of rh-IL-12 for 20hrs, and then incubated with C. neoformans for 48hrs (T48) at E: T ratio 100:1. CFU were determined at 0hr (starting inoculum) and 48hr. Data shown, n= 7 subjects for both healthy and patients on ART (+ART), and n=4 subjects for patients not on ART (-ART). One way ANOVA with Bonferroni correction was used for statistical significance. **p< 0.01, ***p< 0.001, ns (not significant).
4.2 IL-12 restores granule-dependent NK cell anticytocal activity of HIV-infected patients

NK cells kill *Cryptococcus* by degranulation of secretory lysosomes that contain the effector molecule perforin (77). Having demonstrated that IL-12 restored the anticytocal activity, I sought to determine whether a granule-mediated mechanism were responsible. To address this question, NK cells were depleted of granules using strontium chloride (SrCl2). As expected, based on previous results from our laboratory, SrCl2 treatment reduced the enhanced anticytocal activity of NK cells from healthy subjects. Additionally, SrCl2 treatment reduced the enhanced anticytocal activity IL-12-treated NK cells from HIV-infected patients (Figure 2.2A), suggesting that IL-12 restored anticytocal activity of NK cells from HIV-infected subjects via a granule-dependent mechanism. This observation suggested that one or more of the checkpoints in the NK cell cytotoxicity pathway might be the target of IL-12. I therefore sought to determine the effect of IL-12 on the ability of NK cells from HIV-infected subjects to release perforin in response to *Cryptococcus*. To investigate the effect of IL-12 on NK cell release of perforin, from HIV-infected patients, I treated NK cells from healthy donors, and HIV-infected patients with recombinant human IL-12 and assessed their ability to secret perforin in response to *C. neoformans* stimulation. IL-12 treated NK cells from HIV-infected patients showed a significant, 300% increase in perforin release in response to *C. neoformans* compared to NK cells from HIV-infected patients not treated with IL-12 (Figure 2.2 B). In contrast, there was only a 60% increase in perforin release in NK cells from healthy subjects (Figure 2.2 B). The increase in perforin release correlated with increase killing of *Cryptococcus* by NK cells from HIV-infected patients (Figure 2.1 A&B).
Figure 2.2: IL-12 restored defective granule-mediated perforin release in response to *C. neoformans* by NK cells from HIV-infected patients.

A). NK cells from healthy or HIV-infected patients were pretreated with IL-12 for 18h and then incubated in the presence or absence of 10µm SrCl₂ for 24 hr. Treated NK cells were incubated with *C. neoformans* and antifungal activity was determined by CFU. Data shown is the mean of 4 replicates. The experiment was repeated with similar results. B). NK cells from healthy or HIV-infected patients were pretreated with IL-12 for 18h and then incubated in the presence or absence of 10µm SrCl₂ for 24 hr. Viability of cells were assessed by trypan blue exclusion. The experiment was repeated with similar results. C). NK cells from healthy donors and HIV-infected patients were pretreated with rh-IL-12 for 18 hrs. The stimulated perforin release in response to *C. neoformans* after 1 hr was assessed by ELISA, n= 4 subjects. One way ANOVA with Bonferroni correction was used for statistical significance. **p< 0.01, ***p< 0.001. Data is the mean ± SEM.
4.3 IL-12 treatment increased intracellular perforin expression in NK cells from HIV-infected patients

Having demonstrated that IL-12 restored killing via a granule-mediated mechanism and restored perforin release, I considered two possible reasons for the effect. The first possibility was that IL-12 treatment restored the defective perforin stores in NK cells from HIV-infected patients (Figure 1.3). Alternatively, the defective polarization and degranulation of perforin (Figure 1.4) might be restored with IL-12 treatment. To investigate if IL-12 enhanced the intracellular expression of perforin in NK cells from HIV-infected patients, I treated NK cells from healthy donors, and HIV-infected patient with recombinant human IL-12 and assessed intracellular perforin content by flow cytometry. The level of perforin was higher in NK cells from healthy donors and increased minimally when treated with IL-12 (Figure 2.3 A&C). By contrast, levels were lower in NK cells from HIV patients, but induced to a much higher level with IL-12 (Figure 2.3 B&C). This observation suggest that the defective perforin expression in NK cells from HIV-infected patients may account for the defective NK killing of *Cryptococcus*, and that the defect can be restored with IL-12.
A

MFI: 152
HEALTHY+IL-12

MFI: 148.5
HEALTHY

Isotype

B

MFI: 68.4
HIV

MFI: 127
HIV+IL-12

Perforin-FITC

C

Intracellular Perforin (MFI)

**

HEALTHY

HEALTHY+IL-12

HIV

HIV+IL-12

*
Figure 2.3: IL-12 induced higher intracellular perforin expression in NK cells from HIV-infected subjects.

A). NK cells from healthy donors were freshly isolated, treated with or without IL-12, made permeable, labelled with anti-perforin antibody, and analyzed by flow cytometry. Data is representative of 3 experiments. B). NK cells from HIV-infected patients were freshly isolated, treated with or without IL-12, made permeable, labelled with anti-perforin antibody, and analyzed by flow cytometry. Data is representative of 3 experiments. C). Geometric mean fluorescent intensity of perforin labelling in IL-12 treated NK cells from healthy donors and HIV patients (n=3 subjects). One way ANOVA with Bonferroni correction was used for statistical significance. *p< 0.05, **p< 0.01. Data is the mean ± SEM.
4.4 IL-12 restored perforin polarization of NK cells from HIV patients to *C. neoformans*

Having demonstrated that IL-12 increase in the perforin stores in NK cells from HIV-infected subjects, I wondered whether IL-12 would also restore the defect in granule polarization I had previously observed (Figure 1.4B). To investigate if IL-12 restored defective perforin polarization in NK cells from HIV-infected patients, we treated NK cells from HIV-infected patients with recombinant human IL-12 prior to addition of *Cryptococcus*. Images demonstrated that perforin-containing granules were in closer proximity to the synapse with *Cryptococcus* in IL-12-treated NK cells from HIV-infected patients compared to untreated NK cells from HIV-infected patients. Quantitative image analysis showed that perforin-containing granules were closer to the synapse with *C. neoformans* in IL-12-treated NK cells (Figure, 2.4 B&C) compared to untreated NK cells from HIV-infected patients (Figure 5 A&C). Increased polarization is consistent with the enhanced perforin release by IL-12 treated NK cells from HIV-infected cells in response to *C. neoformans* challenge and provided an additional mechanism by which IL-12 restored killing by NK cells from HIV-infected patients.
A

PHASE  PERFORIN  HIV  DAPI  MERGE  PHASE+ MERGE

6 µm 6 µm 6 µm 6 µm 6 µm

B

PHASE  PERFORIN  HIV+ IL-12  DAPI  MERGE  PHASE+ MERGE

6 µm 6 µm 6 µm 6 µm 6 µm

C

DISTANCE OF PERFORIN TO C. neoformans (µm)

***

HIV  HIV+IL-12
Figure 2.4: IL-12 restored polarization of perforin in NK cells from HIV patients to *C. neoformans* target.

NK cells from HIV-infected patients were treated with or without IL-12 for 20 hr and challenged with *C. neoformans neoformans* at an E: T ratio of 1:2 for 1 hr. The NK cells were labeled with anti-perforin Ab (green), and DAPI for nucleus (blue). A). Localization of perforin in non-IL-12 treated NK cells from HIV-infected patients bound to *C. neoformans* at 60X magnification. The traced outline indicates the position of *C. neoformans* in all panels. B). Localization of perforin in IL-12 treated NK cells from HIV patients bound to *C. neoformans* at 60X magnification. The traced outline indicates the position of *C. neoformans* in all panels. C). The distance of perforin to *C. neoformans* in NK cells from healthy donors and HIV patients was assessed using Volocity software (see methods). Each data point is the mean distance of 15 to 30 granules per cell. Data shown is the mean of N=43 untreated or IL-12-treated NK cells that had formed conjugates of 1 donor in each group. The experiment was repeated with different subjects with similar results. Unpaired t tests (two-tailed) with Welch correction was used to determine statistical significance. ***p< 0.001. Data is the mean ± SEM.
4.5 IL-12 restored defective binding of NK cells from HIV patients to *C. neoformans*

To investigate if IL-12 restored the ability of NK cells from HIV-infected patients to bind *C. neoformans*, NK cells from healthy donors and HIV-infected patients were treated with or without IL-12 and conjugates with *C. neoformans* were assessed by flow cytometry. IL-12 treated NK cells from HIV-infected patients showed a significant increase in the percentage of NK cells that bound to *C. neoformans* compared to NK cells from healthy subjects (Figure 2.5A) and (Figure 2.5 B) respectively. This observation supported the notion that IL-12 enhanced the expression of a receptor required for binding to *Cryptococcus* in HIV-infected patients.
A

C. neoformans Binding (%)

Time (mins)

B

C. neoformans Binding (%)

Time (mins)
Figure 2.5 Defective binding of NK cells from HIV patients to *C. neoformans*, is restored with IL-12-treatment.

A). NK cells from HIV patients were treated with or without IL-12 for 18 hrs, and cocultured with *C. neoformans* for different lengths of time (n=4 subjects). B). NK cells from healthy donors were treated with or without IL-12 for 18 hrs, and co-cultured with *C. neoformans* for different lengths of time (n=3 subjects). Conjugates were detected when both red and green were detected in the same event by flow cytometry. Unpaired t tests (two-tailed) with Welch correction was used to determine statistical significance **p< 0.01, ns (not significant).**

Data is the mean ± SEM
4.6 IL-12 restores defective binding of NK cells from HIV-infected patients to *Cryptococcus* by upregulating NKp30 expression

The activation natural cytotoxic receptor (NCR), NKp30 mediates direct cryptococcal recognition and killing by NK cells (111). I therefore sought to determine whether NKp30 plays a role in NK cell antifungal activity of HIV-infected patients. NK cells from HIV-infected patients showed significantly lower surface expression of NKp30 fluorescence as compared to healthy subjects (Figure 2.6 A), which correlated with the reduced anticryptococcal activity (Figure 1.1A), and aberrant perforin release (Figure 1.2B). Upon IL-12 treatment, NK cells from HIV-infected patients showed a significant increase in NKp30 expression as demonstrated by flow cytometric analysis (Figure 2.6 B). This observation supported the notion that IL-12 enhanced the expression of a receptor required for binding to *Cryptococcus* in HIV-infected patients. To determine whether the gain in NKp30 expression was responsible for the improved binding of NK cells from HIV-infected patients after IL-12 treatment (Figure 2.5A), IL-12 treated NK cells from HIV-infected patients were treated with or without anti-NKp30 blocking antibody, prior to assessing their ability to bind *C. neoformans*. Blocking NKp30 significantly reduced the percentage of conjugates formed by IL-12 treated NK cells from HIV-infected patients (Figure 2.6C). These observations provided evidence that reduced binding of NK cells from HIV-infected patients to *C. neoformans* was restored with IL-12 via expression of NKp30. To investigate the possibility that IL-12 might enhance binding as an off target effect with no effect on cryptococcal killing, I blocked the NKp30 receptor following IL-12 treatment with polyclonal anti-NKp30 blocking antibody. The polyclonal anti-NKp30 significantly inhibited IL-12 enhanced killing of *Cryptococcus* by NK cells from HIV-infected patients (Figure 2.6D),
suggesting that NKp30 binding to Cryptococcus is required for NK cell killing of the fungi organism.
Figure 2.6 Defective binding of NK cells from HIV patients to *C. neoformans*, was restored with IL-12 via increased expression of NKp30.

A). Cell surface expression of NKp30 in freshly isolated NK cells from healthy subjects and HIV-infected patients (n=5 subjects). Unpaired t tests (two-tailed) with Welch correction was used to determine statistical significance B). Change in NKp30 labelling on NK cells from both healthy and HIV-infected patients with or without IL-12 treatment (n= 8 subjects). Values = Geo MFI (post-IL-12)/Geo MFI (pre-IL-12). C). NK cells from HIV-infected patients were treated with or without IL-12 for 18 hrs. The cells were then preincubated with polyclonal anti-NKp30 blocking antibody, isotype control or without antibody treatment for 1 hr, prior to assessing conjugate formation with *C. neoformans*. Conjugates were detected when both red and green were detected in the same event by flow cytometry. Experiment was repeated with similar results. D). NK cells from HIV patients donors were treated with or without IL-12 for 18 hrs. The cells were then preincubated with polyclonal anti-NKp30 blocking antibody, isotype control or without antibody treatment prior to assessing anticytotoxic activity. Data shown are representative of three experiments. One way ANOVA with Bonferroni correction was used for statistical significance.

*p< 0.05, **p< 0.01, ***p< 0.001, ns (not significant). Data is the mean ± SEM.
4.7 Chapter Summary

In summary, this chapter establishes that the defects in the NK cell cytotoxic pathway resulting in the defective anticryptococcal activity could be reversed with IL-12 treatment. It demonstrates that IL-12 treatment induced higher perforin expression in NK cells from HIV-infected patients, restored the defective ability to polarize perforin to the NK cell-fungal synapse, and restored defective degranulation and perforin release in response to *C. neoformans* challenge. Additionally, this chapter demonstrates that IL-12 increased the expression of NKp30 on NK cells from HIV-infected patients, and NKp30 is required for cryptococcal binding. Blocking NKp30 inhibited killing of *Cryptococcus*. Thus, IL-12-treatment can restore the multiple defects in the cytolytic machinery of NK cells from HIV patients, which consequently restores the defective anticryptococcal activity of NK cells from HIV-infected patients.
5.1 General discussion

Cryptococcal infection has emerged as one of the most important cause of morbidity and mortality in immunocompromised patients especially in HIV-infected patients, and it is an AIDS-defining illness (64). In high HIV prevalent regions in sub-Saharan Africa, cryptococcal meningitis is the leading cause of meningitis and more common than *Neisseria meningitides* and *Streptococcus pneumonia* (37). Since the introduction of combination antiretroviral therapy, there have been a dramatic improvement in the life expectancy among HIV-infected patients (152). Although ART has revolutionized the quality of life of HIV-infected patients, HIV-infected patients have increased risk of morbidity and mortality when compared to uninfected individuals (232). Indeed, despite the availability of ART, about a million cases of cryptococcal meningitis occur annually with a mortality of 68% within the first 3 months of diagnosis (2). Though there is active anticryptococcal therapy, the high toxicity of these drugs as well as high frequency of relapse among HIV-infected patients (41), underscores the need to for safer alternative treatments such as immunotherapy. NK cells have emerged as important host anticryptococcal immune response in addition to other immune cells (101, 102). Studies have demonstrated direct killing of *C. neoformans* by NK cells (106-108). However, this response is defective in HIV-infected individuals but can be rescued with IL-12 treatment (114). The mechanism underlying this defect and how IL-12 restored the defect is unknown. The goal of this thesis was to decipher the defects in the NK cell direct anticryptococcal activity and how IL-12 restores these defects in HIV-infected patients, hoping to provide insights into the
mechanisms involved in the defect and restoration with IL-12 treatment, which may facilitate the development of immunoregulatory drugs that can restore these defects.

5.2 Functional defects of NK cells from HIV-infected patients

In this study, I have made 5 key observations about defective fungal killing by NK cells from HIV-infected patients; these NK cells had: (1) defective perforin release. (2) Low perforin expression, (3) defective polarization of perforin to the fungal synapse, (4) defective binding in response to C. neoformans stimulation, (5) IL-12-treatment restored these defects in microbial cytotoxicity.

The hallmark of HIV infection is the progressive deterioration of the host immune system initiated by the depletion of CD4+ T-cells with a resultant risk of opportunistic infections and death (233). Antiretroviral therapy restores some aspects of host immune system by decreasing viral load and significantly increasing CD4+ T-cell number (156). Despite the effect of ART on the improved health of HIV-infected patients, several studies have demonstrated that, ART partially restores immune functions of HIV-infected patients. Indeed, long-term ART has been shown to decrease HIV-1-specific CD4+ lymphocytes (234). Further, humoral immune responses to HIV as well as HIV-1-specific CD8+ cytotoxic T-cell responses decline in chronically infected individuals treated with ART (159, 235), suggesting that while ART effectively suppresses viral replication, it may suppress host immune response to HIV. Importantly, there have been no decline in some HIV-associated malignancies despite ART (236), suggesting that important immune cells such as NK cells that are vital in host anti-tumor responses are defective.
Several studies have shown phenotypic and functional defects of NK cells from HIV-infected patients, including aberrant antibody-dependent cellular cytotoxicity (ADCC), high expression of inhibitory natural killer receptors (iNKRs), low levels of NCRs, and reduced tumor cytotoxicity (167, 168, 237, 238). Some studies have implicated a switch in NK cell differentiation from the more cytotoxic phenotype to the less cytotoxic phenotype (172) as the cause of NK cell defects in HIV-infected patients. Other studies have demonstrated that excessive activation of NK cells leads to functional defects as a result of exhaustion (239) in HIV-infected patients. However, whether NK cells from HIV-infected patients on ART had defective antimicrobial activity was unknown. My observations that NK cells from HIV-infected patients had defective direct anticryptococcal activity contribute to this body of literature by demonstrating a functional defect in NK cells that can be restored by IL-12 that was unlikely to be due to a change in differentiation or exhaustion.

5.3 Defects in the cytolytic pathway of NK cells from HIV-infected patients

Only a few studies have investigated binding, and granule trafficking as a possible explanation for defective tumor cytotoxicity by NK cells from HIV-infected patients. These studies demonstrated that NK cells from HIV-infected patients had decreased ability to form conjugates with K-562 and U-937 tumor cell lines (134, 240). It has been reported that NK cells from HIV-infected cells have defective ability to rearrange tubulin after forming conjugates with tumor cells which is critical in tumor killing (134), which leads to the aberrant cytolytic capacities. In addition to studies that show defects in microtubule rearrangement, my results suggest that the aberrant NK cytotoxicity of HIV-infected patients is as a result of a defect in granule mobilization and deployment to target cells. Previous studies from our lab have
implicated the cytolytic machinery in NK cell-mediated antifungal activity. Previous studies have demonstrated that NK cells and T-cells form conjugates with *C. neoformans*, and that contact is required for anticryptococcal activity (77, 241, 242). Other studies have demonstrated that killing of several fungi by NK cells is cytolytic granule dependent (77, 88, 95). Inhibition of perforin but not granulysin by small interfering RNA abrogated NK cell anticryptococcal activity (77), suggesting that perforin but not granulysin is required for direct NK cell anticryptococcal activity. Further, perforin-mediated antifungal activity is not exclusive to *C. neoformans* but against multiple fungi pathogens including *A. fumigatus*, and *Rhizopus oryzae* (94, 140). Indeed, enriched human NK cells lost their anticryptococcal activity after monensin treatment, which is a known inhibitor of golgi transport (241), suggesting the importance of granule movement in the NK anticryptococcal mechanism. Importantly, it has been demonstrated that exocytosis of cytolytic granules are required by NK cells to kill *C. neoformans* (107). However, whether polarization of lytic granules is required for NK cell-mediated anticryptococcal activity was unknown. My results provide strong evidence that polarization of perforin is required for NK cell-mediated anticryptococcal activity, as NK cells from HIV-infected patients do not polarize perforin, release lower amount of perforin and do not kill, while NK cells from healthy subjects or IL-12-treated NK cells from HIV-infected patients polarize their lytic granules, release higher perforin and are able to kill. While there is a possibility that perforin could be released independent of polarization (142), it is most likely that polarization is required for targeted release and killing, and therefore polarization and defective release are linked. In addition to defects in polarization, there was also lower perforin expression, and that restoring perforin content with IL-12 was associated with restoration of killing. It is possible that lower perforin
expression could lead to lower perforin release, suggesting that defective perforin release is the consequence of both defective polarization and lower perforin expression.

Several studies have demonstrated defective NK receptor expression in HIV-infected patients (167, 243, 244). These receptors are important for target recognition and activation of NK cells, including cytotoxic activities. Indeed, the natural cytotoxic receptors (NCRs) primarily bind to target cells and mediate NK cell activating signals that lead to cytotoxicity (245). The observations that lower expression of NKp30 on NK cells from HIV-infected patients correlated with aberrant anticyryptococcal activity (111), as well as less binding to Cryptococcus add to this body of literature, demonstrating a functional consequence of this defect on antifungal activity. Indeed, the observation that IL-12 can correct the low NKp30 expression that consequently corrects binding and anticyryptococcal activity expands our understanding of NK-mediated antimicrobial activity in HIV-infected patients, and provides a window of opportunity to translate this into a potent therapeutic target. While binding is a prerequisite for signal transduction leading to polarization, my results suggests that binding and perforin polarization are independent defects as the residual population of NK cells from HIV-infected patients that did bind to C neoformans failed to polarize perforin to the target. These observations suggest that there are multiple defects in the cytolytic machinery of NK cells from HIV-infected patients. These defects may have implications for other NK cell responses, such as NK cell antitumor activity and antiviral responses (246, 247).

5.4 Implications of defects in NK cell cytolytic machinery on tumor killing

NK cell cytolytic response is one of the most important mechanisms of host antitumor defense. Indeed NK cells from HIV-infected patients have been shown to be defective in the
killing of tumor cells (237, 248). However, only a few studies have explored the cytolytic pathway of NK cell mediated tumor killing in HIV-infected patients. LFA-1 which plays essential roles in NK cell adhesion, conjugate formation, and polarization of cytotoxic granules in tumor killing was demonstrated to be decreased on NK cells from HIV-infected patients (170). Though decreased LFA-1 expression is suggestive of impaired conjugate formation, it is unknown whether this defect affects conjugate formation of NK cells from HIV-infected patients with tumor cells. My results showing that decreased NKp30 correlates with less binding of NK cells to *C. neoformans*, suggests that decreased LFA1-1 expression might affect conjugate formation between NK cells from HIV-infected patients and tumor targets. In one study, it was demonstrated that NK cells from HIV-infected patients failed to rearrange tubulin after binding to K562 tumor targets (134), suggesting the possibility of impairment in NK cell cytolytic granules transport to the immunological synapse, however, whether defects in tubulin rearrangement affects cytolytic granule polarization to target cells was unknown. My results demonstrating that perforin-containing granules failed to polarize to *C. neoformans* targets expands our knowledge on the mechanisms underlying the defects in the cytolytic machinery of NK cells from HIV-infected patients, suggesting that impaired perforin polarization may in part be due to defects in microtubule rearrangement. My observation of defects in the cytolytic pathway could explain why despite ART, the frequency of HIV-associated malignancies have not declined (236), suggesting that the persistence of these defects extends beyond ART and NK-cell antimicrobial defense.
5.5 Importance of IL-12 in host antifungal immune response

My observation that IL-12 restored the defective anticryptococcal activity of NK cells from HIV-infected patients highlights the importance of IL-12 in host NK cell immune response. Importantly, HIV-infected patients have aberrant IL-12 production in response to protozoal and bacterial stimuli (249, 250). The mechanism involves the suppression of IL-12 p35 production resulting in defective release of the biologically active IL-12 heterodimer in monocytic lineages by HIV-vpr (218). IL-12 has been demonstrated to increase perforin in NK cells with a consequent increase in NK cell tumor cytotoxicity (205, 251). Indeed, it has been shown that IL-12 promotes perforin expression through the direct binding of STAT4 to the perforin gene promoter (252). However, whether IL-12 promotes perforin expression in NK cells from HIV-infected patients was unknown, my results add to this this body of knowledge demonstrating that IL-12 promotes perforin expression in NK cells from HIV-infected patients. Several studies have demonstrated the importance of IL-12 in the treatment of opportunistic infections as well as tumors (253, 254). However, the toxicity of IL-12 treatment poses a therapeutic limitation. Despite its toxicity in patients, IL-12 therapy has been effectively administered to AIDS patients with Kaposi’s sarcoma, for which other therapies have failed (223, 224). Indeed, IL-12 administered in the absence of antiretroviral therapy to Indian Rhesus macaques infected with simian immunodeficiency virus led to restoration of NK lytic functions (226). Further, IL-12 has been demonstrated to be a potent cytokine in host antifungal immune response. IL-12 synergizes with antifungal agents such as fluconazole to reduce yeast burden in the brain, lung and liver, as well as in conjunction with amphotericin B against *H. capsulatum* infection in mice (255, 256). Further, IL-12 can protect Balb/c mice susceptible to *Coccidioides immitis* infection by switching the immune response from the unprotective Th2 to the protective Th1 response (254).
Interestingly, IL-12 has been shown to be essential in NK cell anticryptococcal activity. Mice with targeted deletion of IL-12, infected with *C. neoformans* had higher numbers of the fungi in both brain and lung compared to controls (257). Ex-vivo treatment of NK cells from HIV-infected patients with IL-12 restored their defective anticryptococcal activity (114). New therapeutic approaches have demonstrated successful use of IL-12 in treating leukemias (227, 228), suggesting the possibility of overcoming the toxicity of IL-12 therapy. Because of the systemic toxicity of IL-12, cytokines or combinations of cytokines might be considered, but that the sum of their effect would need to restore perforin content, granule polarization and conjugate formation, since each of these effects is restored by IL-12. My results contribute to the literature by demonstrating that IL-12 is sufficient to restore permanent defects in NK cells from patients receiving ART and establish the mechanism.

5.6 IL-12 signaling pathway and host antimicrobial immune response

Alternate cytokines may provide a less toxic effect to IL-12 if they signal through the same pathway. IL-12 promotes NK cell cytotoxicity by signaling through the Janus family kinases Tyrosine kinase 2 (Tyk2) and Janus kinase 2 (Jak2), which predominantly activates STAT4 (258). Indeed, STAT4 has been demonstrated to be critical in host antimicrobial and immune responses. It was demonstrated that STAT4 knock out mice were most susceptible to mycobacterial infection compared to wild type and STAT6 knock out mice (259). Further, STAT4 deficient mice have been shown to be susceptible to several parasites including *Leshmania major, T. gondii* and *Babesia* (260-262). The importance of STAT4 in host antimicrobial immune responses suggests that cytokines that signal through this pathway could serve as potential therapeutic agents. Although IL-12 is the main activator of STAT4 (258), other
cytokines have also been demonstrated to activate STAT4. For instance, IFN-γ that is known to predominantly activates STAT1 (263), has also been demonstrated to activate STAT4 (264). This may explain why unlike IL-12, IFN-γ treatment of NK cells from HIV-infected patients promoted partial restoration of defective anticryptococcal activity (114), suggesting that IFN-γ could restore some of the defects in the NK cytolytic machinery of HIV-infected patients. Other cytokines that have been shown to activate STAT4 include type 1 IFNs, IL-2, IL-15, IL-21 and IL-23 (258, 265-267). Although the consequences of STAT4 stimulation by these cytokines in the NK cytolytic machinery are unknown, they however may serve as possible substitutes for IL-12 in ameliorating the toxic effect of IL-12-treatment. The importance of the JAK-STAT signaling in host antimicrobial defense suggests that caution must be taken in clinical use of broad-spectrum JAK inhibitors such as tofacinitib, ruxolitinip and oclacitinib (268, 269). Indeed, opportunistic infections including Mycobacterium tuberculosis, Pneumocstis jirovecii and bacteria pneumonia have been reported in patients receiving tofacinitib (JAK1, JAK2, and JAK3 inhibitors) (270, 271). This provides clinical evidence of the importance of the JAK-STAT pathway in host antimicrobial defense. These observations notwithstanding, the current study expands our knowledge on the defective NK cytotoxic activity of patients receiving ART.

5.7 Translational implications for disease progression in HIV-infected patients

My observation may also have clinical implications. The finding that NK cells from HIV-infected patients had defective anticryptococcal activity during ART is consistent with prior observations that ART restores CD4+ T-cell numbers and DCs activities, but not NK cells (166, 167). I speculate that the defect in NK cells is permissive, but not sufficient for cryptococcal infection. It has been demonstrated that HIV-infected patients receiving ART have high
cryptococcal antigenemia (272), suggesting they have acquired a subclinical infection. It is possible that these patients develop subclinical infection and antigenemia because of impaired NK cells, but other immune mechanisms such as T-cell activities keep the pathogen in check and prevent dissemination. Indeed, the persistence of NK cell defects during ART suggests irreparable damage to the immune system which does not recover with ART. It is possible that starting ART early could prevent the damage to the NK cell compartment and further damage to the immune system (273, 274). Further study will be required to determine whether immune reconstitution at the stage of asymptomatic antigenemia reduces the incidence of cryptococcosis. The initiation of ART is not without pitfalls. Unfortunately, about 7-50% of HIV-infected patient starting ART develop clinical worsening immune reconstitution inflammatory syndrome (IRIS), that is characterized by localized and systemic inflammatory symptoms (275, 276). Therefore alternative therapy such immunotherapy with cytokines that restore these defects will be desirable.

**Limitations of study**

The outcome of this study has unraveled important findings that have both biological and clinical implications. However, there are limitations to this study. The influence of CD4+ T-cell numbers on NK cell antifungal activity was not studied, for example, whether patients with high CD4+ T-cell count correlated with increased NK cell antifungal activity was not studied. It has been demonstrated that tumor cytotoxicity of NK cells from HIV-2 infected patients correlates with CD4+ T-cell counts (277), suggesting that CD4+ T-cell count may influence NK cell antimicrobial activity. Further, variability in patient and control subjects is another limitation. As an example, the effect of short or long term ART on NK cell antifungal response was not addressed in this study. In addition, the wide age range (24-55 years) of participants in the study
introduces variability in the observations made in this study. Because aging is associated with immunosenescence (278), it will be informative to study the effect of age on NK cell direct antimicrobial responses in healthy and HIV-infected patients. Although patients with known coinfections were excluded from this study, it was still unknown whether patients had other comorbidities such as inflammatory bowel disease or any other non-infectious morbidities. Finally, various techniques are used in this study however, other alternative approaches to answering the same experimental questions are lacking. For instance, Flowcytometric analysis was used to quantify intracellular perforin expression by NK cells. Alternatively, immunoblotting techniques or ELISA could be used to answer the same experimental question.

5.8 Future directions

Although this study has contributed immensely to our understanding of NK-cell anticytolic activity of HIV-infected patients, by unraveling important mechanisms underlying their defective NK-cell direct anticytolic killing and the mechanisms underlying how IL-12 restores these defects, there however remain some questions yet to be answered. Particularly, how HIV induces specific defects in NK cells of HIV-infected patients remain unknown. Considering the fact that less than 2% of NK cells may productively get infected with HIV (279), it is highly unlikely that the defects in the cytolytic pathway of NK cells are as a result of direct HIV infection of these cells. However, it will be worth investigating the effects of HIV-accessory proteins on the NK cell cytotoxic pathway. Indeed, the HIV-negative factor (Nef) which is expressed earliest in HIV infection, is an HIV-accessory protein known to impair the activity of various immune cells. Nef induces lysosomal degradation of CD4-receptors on T-cells (280). Importantly, Nef has been shown to reduce MHC-1 expression on cells by re-routing
MHC-1 from the trans-Golgi network to lysosomes (281). It is possible that Nef interferes with Golgi transport of NKp30 leading to lower expression in HIV-infected patients. Further, it is possible that HIV-accessory proteins such as Nef interfere with microtubule transport of cytolytic granules to target cells, which may provide an explanation for why perforin does not polarize to cryptococcal target in HIV-infected patients. Indeed the HIV viral protein (Vpr) is transported to the MTOC in a microtubule-dependent manner (282), suggesting that HIV has the potential to hijack this pathway. In addition, it is unknown how HIV-infection leads to lower perforin expression. I speculate that HIV-accessory proteins interfere with signaling pathways involved in perforin synthesis. It is also possible that HIV-accessory proteins prevent conversion of inactive perforin to the active form or promote degradation of perforin. Intracellular labelling of Nef in NK cells by flow cytometry or by immunoblotting, or fluorescence imaging will be an important first step in determining whether Nef binds to targets in NK cells from HIV-infected patients. Because NK cells are not productively infected with HIV, labelling of Nef in these patients could prove challenging. Alternatively, NK cells from healthy donors can be treated in vitro with Nef proteins, and determine if Nef interferes with the NK cell cytolytic pathway. Flow cytometry can be used to determine if treatment of NK cells from healthy subjects with Nef results in down regulation of NK receptors including NKp30, and perforin expression. Immunofluorescence microscopy could be used to determine if Nef prevents the transport of NKp30 or perforin to the cell surface and cytolytic granules respectively. This technique has been successfully used to show that Nef inhibits the surface expression of M-CSF receptor on macrophages by inducing its localization in the Golgi (283). Additionally, this work did not fully address whether IL-12 induces higher active perforin by upregulating perforin gene transcription or by promoting the transport of inactive perforin from the Golgi to the lytic granules. Up
regulation of the perforin gene can be determined by Reverse transcriptase polymerase chain reaction (RT-PCR). Immunofluorescence microscopy could be used to determine if IL-12 promotes the transport of perforin from the Golgi to the lytic granules. Further, immunofluorescence imaging techniques could be used to determine whether the defective polarization of perforin is a consequence of defects in cytolytic granule transport motors such as kinesin-1 (128), and whether IL-12 restored these defects.

In conclusion, the current studies show that defective binding, polarization of perforin and release of perforin by NK cells from HIV-infected patients is responsible for the aberrant anticryptococcal activity, and that IL-12 therapy can correct these defects, suggesting that the defect in NK cells from HIV-infected patients are functional rather than exhaustion. This study further enhances our understanding of the NK cell anticryptococcal activity of HIV-infected patients, and has hopefully provided targets for therapeutic interventions to rescue the defects in the direct NK cell anticryptococcal activity.
REFERENCES


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