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NK cells play important roles in immunity against pathogens and cancer. NK cell functions are regulated by inhibitory and activating receptors binding corresponding ligands on the surface of target cells. During pathological conditions, NK cells were shown to be recruited to the CNS and could impact CNS physiology by killing glial cells and by secreting IFN-g. Astrocytes are intimately involved in immunological and inflammatory events occurring in the CNS and reactive astrogliosis is a key feature in HIV-associated neurocognitive disorders (HAND). There is little data on NK cell-astrocyte interactions and ligands expressed on astrocytes that could impact NK cell function. This study aimed to identify NK-associated ligands expressed by human astrocytes that confer this NK-directed cytotoxicity of astrocytes and assay the cytotoxicity differences in presence and absence of HIV 3S peptide.

Using a fusion protein consisting of the extracellular domain of NKp44 fused to Fc portion of human IgG, we determined the expression of a novel ligand for NKp44 (NKp44L) on astrocytes. Incubation of astrocytes with 3S peptide downregulated NKp44L expression on astrocytes implicating protection from NK mediated killing. Thus, our study demonstrated that NKp44 has a protective effect on astrocytes from NK cell mediated killing during HIV infection. Astrocytes could also secrete cytokines that affect the expression of NK receptors on NK cells. We evaluated the expression of receptors on NK cells after co-culture with astrocytes. CD38 expression was increased on primary NK cells after incubation with astrocytes. CD38 is expressed on both NK cells and astrocytes and has an important implication in HIV-1 infection. Blocking CD38 signaling in our studies decreased astrocyte

lysis, suggesting CD38 signaling has important implications in NK-astrocyte interactions. Future studies providing novel insights into the role of NK cells in the pathogenesis of HAND and other brain disorders might result in the development of NK cell based therapies for brain pathologies.

EVALUATION OF NK CELL – ASTROCYTE INTERACTIONS: POTENTIAL ROLE IN HIV-ASSOCIATED NEUROCOGNITIVE DISORDERS AND HIV- ASSOCIATED DEMENTIA

THESIS

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CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1 Human Immunodeficiency Virus-1 (HIV-1)

The human immunodeficiency virus (HIV) global pandemic has been a public health crisis for over three decades, evident from the fact that worldwide some 33 million individuals are infected with the predominant form of the virus, HIV-1. It is estimated that 1.3 million of those infected individuals are living in the United States (UNAIDS report, 2012). HIV-1 primarily infects cells with CD4 cell surface receptors such as macrophages and CD4+ T cells. Infection leads to immunodeficiency and opportunistic infections caused from the progressive depletion of CD4+ T cells [1].

HIV-1 virons are spherical in structure and comprise a viral envelope, enclosing a protein capsid, which encloses the RNA genome (Figure 1.1) [2]. HIV-1 envelope glycoprotein complex gp120/gp41 is drastic for HIV-1 entry into a target cell. Envelope protein gp120 binds sequentially to CD4 and co-receptor for initiating virus entry (Figure 1.2) [2]. CCR5 is the predominate co-receptor in T cells, but there are two other recognized variants. One variant uses CXCR4 for entry [1, 3]. The second variant retains the requirement for both CD4 and CCR5 but has evolved to enter cells with lower expression of CD4 [1]. Binding of gp120 to target cell CD4 surface induces conformational changes in the gp120/gp41 complex that facilitate gp120 interaction with co-receptor [2]. Subsequently, gp41 self-assembles to form a six-helix bundle. Formation of several gp41 six-helix bundles drives the fusion of viral and host membranes

(Figure 1.2) [2, 4]. Highly conserved liner motif NH2-SWSNKS-COOH (3S peptide) of gp41 was found to be localized in a region that plays a vital role in the formation of the six-helix bundle [4-6], and several models propose that this region has contact with the host cell membrane [5, 6]. Conservation of the 3S peptide motif has been reported in at least 77% of the gp41 HIV-1 strains analyzed [6]. This remarkedly conserved 3S peptide plays a critical role and is of vast interest in potential therapies against HIV-1.

Despite 30 years of research, a successful HIV-1 vaccine has not been developed. HIV-1 has the ability to undergo rapid evolution that results in genetic variability and immune escape mutants making it difficult to yield a successful vaccine [1]. The standard and most effective treatment for HIV/AIDs is a drug cocktail consisting of at least 3 different antiretroviral drugs often called highly active antiretroviral therapy (HAART). This drug cocktail combination usually consists of reverse transcriptase inhibitors that inhibit replication of the virus and protease inhibitors that prevent synthesize of viral proteins. The introduction of HAART has significantly decreased the incidence and prevalence of major opportunistic infections in HIV-1 patients and resulted in drastic improvement in survival rates [7, 8]. Up to 15% of HIV/AIDS patients are now over 50 years of age and, with continued treatment, many will reach normal life expectancy [7, 8]. As HIV-1 infected individuals are living longer, factors previously not considered, are now being brought to light. These factors include the effects of aging on HIV-1 infection and HIV-1 infection in the brain [8]. Indeed, HAART has made a significant improvement in the outcome of HIV/AIDs patients, but HAART only slows the course of the disease by controlling viral replication. HAART does not eradicate the virus even after many years, and cellular reservoirs of HIV-1, especially in the brain where anti-HIV drugs cannot be delivered, undoubtedly remain [9, 10].

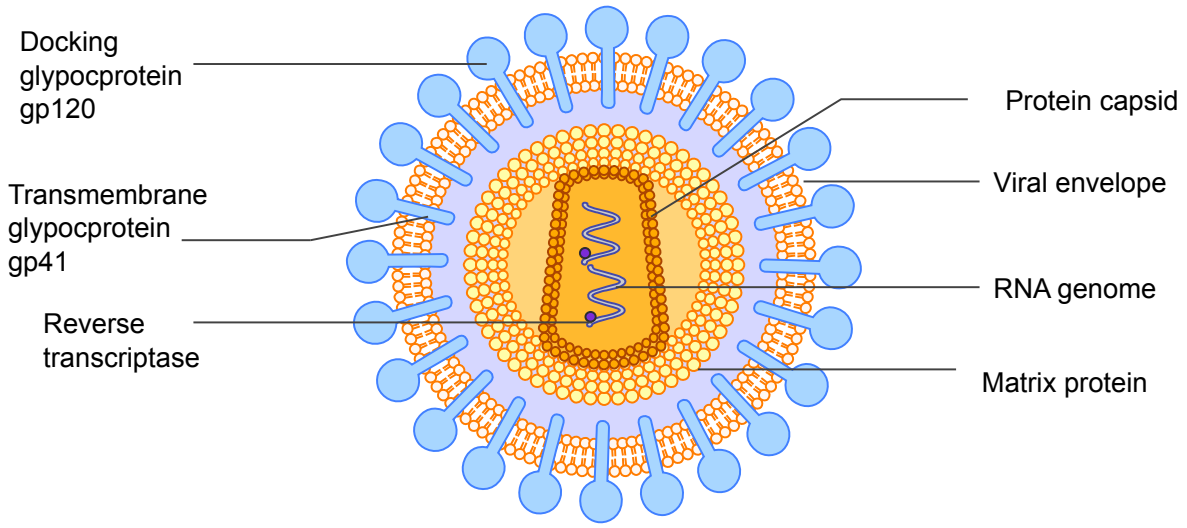

Figure 1.1 Diagram of HIV-1

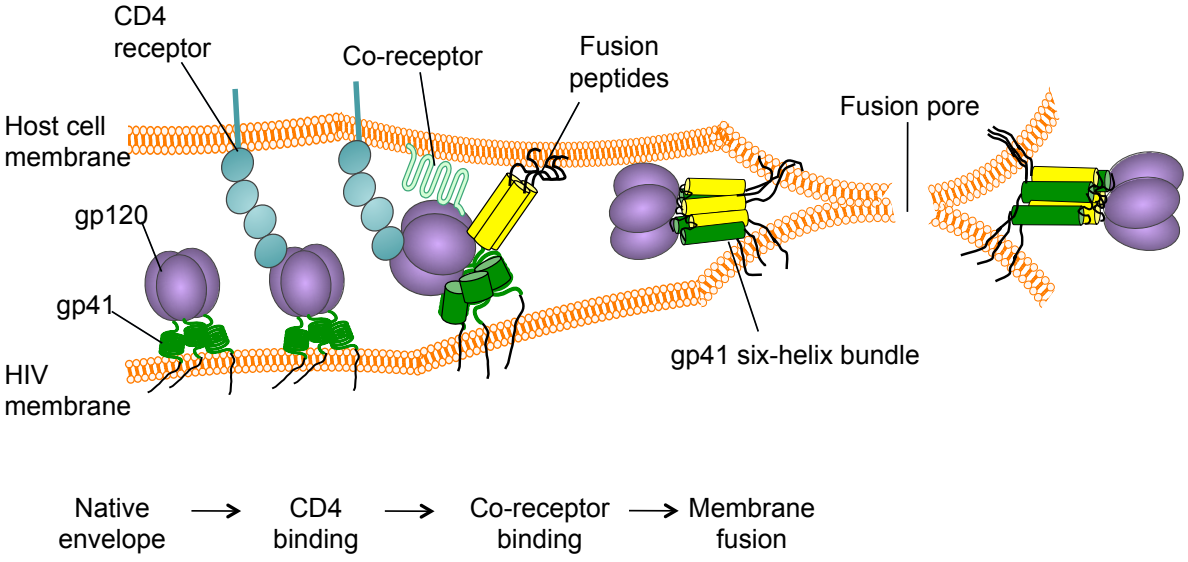

Figure 1.2 Model for HIV-1 entry. The entry process begins with binding of gp120 to its primary receptor CD4. CD4 binding results in conformational changes that allow binding of gp120 to the co-receptor. Co-receptor binding results in the formation of the gp41 six-helix bundle required to drive fusion of the viral and host cell membranes.

1.2 HIV- Associated Neurological Disorders (HAND)

A common cellular reservoir of HIV-1 is the central nervous system (CNS). HIV-1 has the ability to invade the CNS after primary infection and infect CNS resident cells such as macrophages, microglial cells, and astrocytes [9]. HIV-1 infected CNS cells result in inflammatory responses generated in the CNS, leading to long-term neuroinflammation and neuronal damage [11]. This neuronal damage can cause neuropsychological deficits, collectively referred to as HIV-associated neurological disorders (HAND). Symptoms of HAND include: confusion, forgetfulness, behavioral, changes, problems with cognition, and dementia. HIV-associated dementia (HAD) constitutes the most severe form of HAND [12]. The clinical and neuropsychological profile of HAD is generally consistent with a frontal-subcortical pattern of injury [7]. Patients with HAD show marked impairment in cognitive function, especially in learning of new information, information processing, and attention or concentration [13]. Even with the widespread use of HAART, HAND is prevalent in 50% of HIV-1 infected individuals [12, 14]. This high prevalence is due to longer life span of infected individuals and poor penetration of antiretroviral drugs across the blood brain barrier (BBB) [15]. The assessment, pathogenesis, and treatment of HIV-1 in the CNS remain elusive [16], but it is becoming evident that astrocytes, despite only a small percentage being infected contribute to HIV-1 neuropathogenesis [9].

1.3 Astrocytes

Astrocytes are the most abundant cell type in the CNS. They play a vital role in CNS homeostasis and function. Some of their functions include: regulation of glutamate and extracellular ions, support of neuronal energy metabolism by exporting glucose or lactate, and

modification of extracellular matrix [17]. Among their many supportive functions, astrocytes help to maintain the BBB [17]. Astrocytes are intimately involved in immunological and inflammatory events occurring in the central nervous system (CNS), due to their ability to produce and respond to a battery of immunoregulatory cytokines and chemokines such as interleukin (IL) -1β, IL-6, IL-8, IL-10, IL-17, IL-27, tumor necrosis factor (TNF) -α, transforming growth factor (TGF) -β, interferon (IFN)-γ, IFN-β, CCL2, CCL3, CCL5, CXCL10, and CXCL12 [18]. The secretion of these cytokines and chemokines is limited in healthy CNS, but expression of these proteins is seen in Alzheimer's, multiple sclerosis and HAND [18].

Astrocytes are critical for brain homeostasis and for responses to brain injury and infection [19, 20]. Defects in astrocyte functions can lead to neurodegeneration [19, 21]. Astrocytes respond to CNS insults through reactive astrogliosis, the recruitment to proliferation of astroglial cells at injury sites. HIV-1 is a significant modulator of astrocyte proliferation [22, 23] and astrogliosis is a key pathological feature in HAD brains [17, 24]. Apoptotic astrocytes are a feature of reactive astrogliosis and could be an important mechanism of regulation [25]. Astrocytes become more sensitive to apoptosis in the presence of IFN-γ [25] and HIV-1 [22, 23]. Thus, it is becoming more characterized that astrocytes play a role in the neuropathogenesis of HIV-1 infection.

1.4 HIV-1 Infection of Astrocytes

There is growing interest in the potential role of astrocytes in HIV-1-mediated neuropathogenesis [22, 26]. HIV-1 can bind efficiently to astrocytes, but only a small fraction of astrocytes are susceptible to HIV-1 infection [26]. In contrast to highly productive and cytopathic infection in T cells and macrophages, HIV-1 infection of astrocytes is inefficient, of low

productivity, and generally noncytopathic [19]. Previous studies using a pseudotyped HIV-1 virus, found that primary human fetal astrocytes are permissive to productive HIV-1 infection and synthesized HIV-1 viral proteins, and released infectious progeny [19], demonstrating that astrocytes have no intrinsic intracellular restriction to successful HIV-1 replication and the major restriction to HIV-1 infection of primary astrocytes is at virus entry as astrocytes lack CD4 receptor [7, 19]. Although astrocytes lack CD4, they express CXCR4, and under certain circumstances, CCR3 and CCR5, the co-receptors for HIV-1 entry into cells [26, 27].

Recent findings demonstrated that HIV-1 was successfully transferred to astrocytes from infected CD4+ T cells in a cell–cell contact-dependent manner and could result in HIV-1 latency in astrocytes [28]. Latent HIV-1 infection in astrocytes can serve as a hidden reservoir for virus in the brain. The exact roles of the astrocytes in serving as HIV-1 reservoirs in the CNS and their contributions to HAND in the era of HAART remain enigmatic. The elimination of astrocytes latently infected by HIV-1 may be neurologically detrimental.

Despite only a small number of astrocytes being infected, productive infection of HIV-1 in astrocytes still has significant effects on cell physiology and associates with measurable neuropathology in a mouse model [26, 29]. Abundant expression of HIV-1 regulatory proteins in astrocytes is associated with severe to moderate dementia [30]. The binding of HIV-1 to astrocytes independent of infection induces cytokine production and leads to neuroinflammatory responses [26]. HIV-1 binding to astrocytes is sufficient to trigger synthesis of IL-6, a major innate immune effector associated with HIV-1 neuropathogenesis [26]. Previous studies demonstrated that IL-1β and HIV-1 activated astrocytes upregulate CD38 causing an increase in intracellular calcium and disrupt glutamate transport by astrocytes, ultimately resulting in

neuronal damage [31, 32]. This data suggests that astrocytes are exquisitely responsive to HIV-1 and its responses may contribute to HAND.

HIV-1 infection of astrocytes also damages the BBB [33, 34]. HIV-1 infection has been reported to deregulate astrocytic end feet resulting in significant BBB disruption and permeability [34]. Autopsies examining the BBB from HIV-1-infected patients with encephalitis found tight junction integrity was diminished, a fundamental feature of HAND [33, 34]. BBB disruption leads to recruitment of a large number of leukocytes into the CNS, among which are natural killer (NK) cells [35-37]. It is estimated that NK cells comprise 10–20% of CNS infiltrates in experimental autoimmune encephalomyelitis (EAE) models [38, 39].

The cell-cell interactions of NK cells and HIV-1 infected astrocytes are understudied. Because both HIV-1 binding and infection can affect astrocyte function, astrocytes have a strong pathogenic potential for being intimately involved in HAND. Considering the importance of astrocytes for the function of the CNS, future studies evaluating the function of NK cells on HIV-1 infected astrocytes are urgently needed.

1.5 Natural Killer (NK) Cells

NK cells are granular lymphocytes that play a vital role in defense against viral infections and cancer [40-42]. The majority of NK cells are localized in peripheral blood, lymph nodes, spleen and bone marrow but can be induced to migrate toward inflammation site by different chemoattractants [43]. NK cells survey host tissues and kill abnormal cells or virally infected cells [42, 44]. NK cell functions include: release of cytotoxic granules, antibody-dependent cell-mediated cytotoxicity (ADCC), and cytokine production [45, 46]. Cytokines such as IL - 2, IL-12, IL-15, and IL-18 activate and stimulate proliferation, cytotoxicity, and IFN- γ release [46].

These cytokines are released by other cells of the immune system in response to infection and inflammation [46]. NK cells work to control viral infections by secreting IFN-γ and TNF-α [40, 42, 47]. In the event of ADCC, the NK cell recognizes a target cell covered in antibody by utilizing a surface receptor, CD16, which binds the constant heavy chain portion of antibodies known as the Fc fragment [46]. This recognition results in NK cell activation and release of cytotoxic granules such as granzymes and perforin, against the target cell. Upon degranulation, perforin will be released and insert itself in the target cell's membrane forming a pore. Granyzme will then be able to induce apoptosis in the target cell [45].

NK cell recognition of target cells depends on specific receptors that interact with their respective ligands to either stimulate or inhibit NK cell action. It is the sum of signals received from both inhibiting and activating receptors that determines whether the NK cell remains inactive or becomes activated. Inhibitory receptors include the killer cell Ig-like receptors (KIRs) [45, 48]. Inhibitory receptors recognize major histocompatibility complex (MHC) I molecules presenting self antigens on the target cell surface [42, 45, 47]. The 'missing-self hypothesis' postulates that NK cells are activated to kill target cells when the NK cell encounters a host cell that lacks 'self' MHC class I, as shown in the Figure 1.2 [48]. Activating receptors include the natural cytotoxicity receptors (NCRs) and NKG2D [45]. Not all the cellular ligands for NCRs have been identified yet, but studies suggest that not all of them are expressed on normal tissues, and can be induced under certain pathological conditions [6, 45, 48]. When NK cells interact with virus-infected or transformed cells that express ligands for their activating receptors (bottom two panels in Figure 1.2), the NK cell response is regulated by signals received from their inhibitory receptors for MHC class I [42, 47, 48]. Some viruses downregulate MHC class I in the infected cell and can therefore be prone to NK cell killing due to lack of inhibitory signals [41,

42]. For viruses that do not downregulate MHC class I or increase expression of MHC class I in the infected cell, NK cells can kill the infected target cells, provided that signals from the activating receptors can overcome the inhibitory signals [48]. Thus, NK cell killing is regulated by the delicate balance of signals from both activating and inhibitory receptors [42, 48].

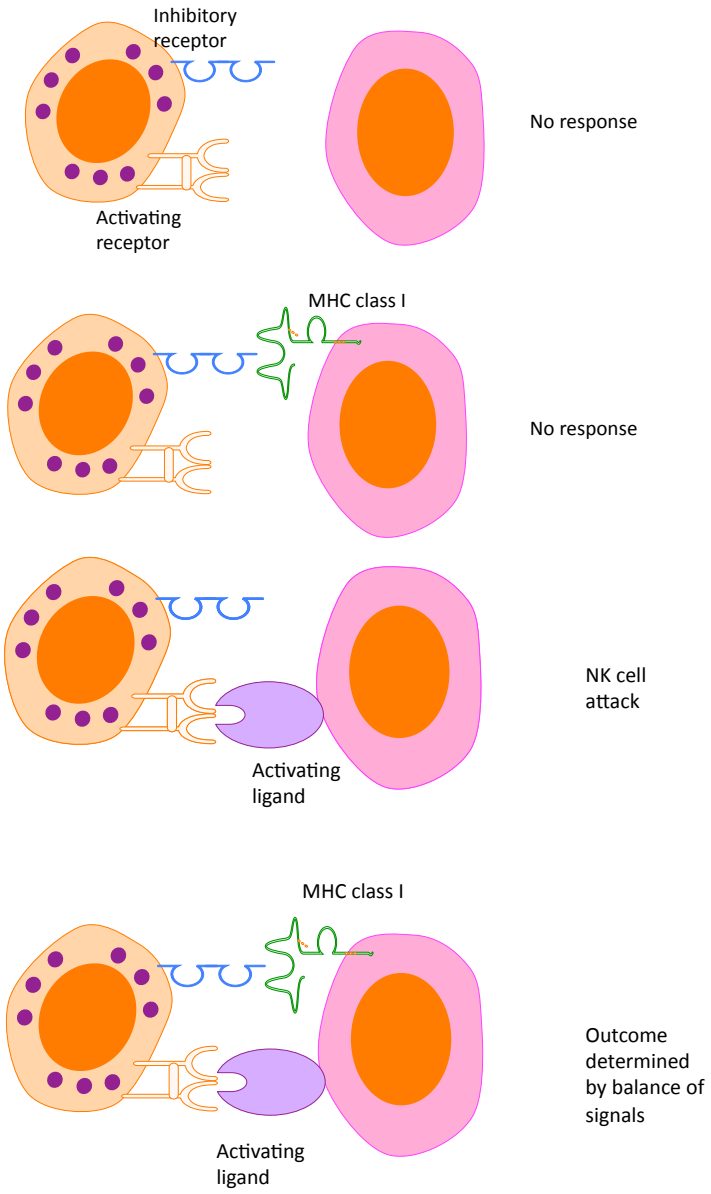

Figure 1.3 NK cell activation is regulated by a delicate balance between signals mediated through NK cell activating and inhibitory receptors.

1.7 Receptors & Ligands Important for NK Cell Function

2B4 and CD48

Previously, our lab cloned the 2B4 (C244, SLAMF4) receptor. 2B4 is a member of the signaling lymphocyte activation molecule family (SLAM) expressed on all NK cells, monocytes and CD8+ T cells [49]. This receptor can either be inhibitory or activating depending on the ligand it interacts with which contributes to the level of NK cell cytotoxicity towards a target cell. 2B4 helps protect against viral infections like Epstein–Barr virus (EBV), herpes simplex virus, and HIV [50, 51]. 2B4 expression on NK cells increases in HIV-1 infected patients followed prospectively during highly active antiretroviral therapy [51]. The expression of 2B4 on $CD8^+$ T cells was also shown to increase during AIDS disease progression [50]. Previous studies in our lab suggest, $2B4^+$ $CD8^+$ T cells play an inhibitory role against constrained HIV epitopes underlying their inability to control the virus during disease progression [50].

The high affinity ligand for 2B4 is CD48. Engagement of 2B4 on NK cell surfaces CD48 can trigger cell-mediated cytotoxicity, IFN-γ and NK cell invasiveness in human cells [52]. During infection the expression of CD48 can be altered. CD48 is found to be downregulated during HIV-1 infection [53]. CD48 was reported to be upregulated in the inflammatory lesions in the brain of multiple sclerosis patients [54]. This upregulation could be due to with release of cytokines that serve to increase CD48 expression leading to NK cell activation and susceptibility to NK-mediated injury [41, 54]. CD48 is highly expressed on the surface of EBV and plays a protective role. However, NK cells from x-linked lymphoproliferative disease patients could not kill EBV cells, suggesting an inhibitory effect [55].

CS1

An additional receptor cloned in our lab, CS1 (CD319, SLAMF7, CRACC), is a member of the slam family expressed on NK cells, B cells, dendrictic cells, and activated T cells [52, 56, 57]. CS1 is a self-ligand, exhibiting homophilic interactions, and has both activating and inhibitory functions in NK cells [58]. In B cells, CS1 induces proliferation and production of autocrine cytokines [59]. In monocytes, CS1 inhibits the production of proinflammatory cytokines [60]. Previous studies report over expression of CS1 in multiple myeloma and treatment of multiple myeloma cells with a monoclonal anti-CS1 antibody inhibits myeloma cell adhesion and induces antibody dependent cell mediated cytotoxicity [61]. Elevated expression of CS1 on B cells is found in systemic lupus erythematosus patients [62]. Thus, CS1 has a wide role in mediating host defenses.

LLT1(CLEC2D)

Lectin-Like Transcript 1 (LLT1), previously cloned in our lab, is expressed on a subset of NK cells, T cells, B cells and all monocytes. NKRP1A (CD161) is the natural ligand for LLT1 and this interaction activates NK cell IFN-γ production but not killing [63, 64]. IFN-γ increases LLT1 expression level on antigen-presenting cells. Studies reported LLT1 cell surface expression on B cells following *in vitro* infection with EBV and HIV-1 [63, 65]. Recent findings report that LLT1 is preferentially expressed on activated DC and B cells, suggesting LLT1 may regulate interactions between NK cells and DC cells during viral infection or interactions between NK cells and activated B cells [66]. TGF-β upregulates the expression of LLT1 in glioblastoma cells, contributing to tumor-associated immunosupression by decreasing NK cells' lytic activity [67].

NKp44 and the ligand for NKp44 (NKp44L)

Natural cytotoxicity receptor NKp44 (CD336) is only expressed on activated NK cells. IL-2 induces the expression of NKp44 on NK cells [54]. NKp44 can be activating or inhibitory depending on the ligand it binds [68, 69]. Strikingly, the ligand for NKp44 (NKp44L) has not yet been detected on circulating cells isolated from healthy individuals, but it is expressed on a large panel of the tumor and transformed cells [70, 71].

The known cellular activating ligand of NKp44 is an isoform of the mixed-lineage leukemia-5 protein (MLL5) [70, 71]. Its activating ligand is expressed in numerous tumor and transformed cell lines, rendering them more sensitive for NK cytotoxicity. Previous studies in our lab identified PCNA/HLA-1 as an inhibitory ligand for NKp44 [68].

NKp44 and NKp44L have strong implications in HIV-1 infection. A substantial percentage of NK cells from HIV-1 patients express the NKp44 [6]. HIV-1 infection has been proven to induce the expression of NKp44L in both infected and uninfected CD4 T cells [6]. Its expression at the surface of CD4+ T cells is induced by a HIV-1 gp41-derived 3S peptide that binds to gC1qR [72]. Higher expression of NKp44L on CD4+ T cells resulted in their lysis by activated NK cells [6]. After HAART, lysis of CD4+ T cells is reduced in association with reduced NKp44L expression [6]. These studies implicate a correlation of NKp44L with both the progression of $CD4^+$ T cell depletion and the increase of viral load suggesting a harmful function.

NKG2D

NKG2D (CD314) is an activating or coactivating receptor expressed on human NK cells, CD8+ T cells, and gamma/delta T cells [73]. NKG2D recognizes several ligands: the MHC I-related chain A and B (MICA and MICB) and UL16 binding protein 1–6 (ULBP1-6) [74, 75]. NKG2D ligand expression is highly restricted in normal tissues but can be can be induced on nontransformed cells by cellular stress and environmental signals including DNA damage and inflammation [73, 74, 76]. HIV-1 vpr protein was reported to induce NKG2D ligands and trigger NK cells to kill autologous HIV-1 infected cells [74], whereas, HIV-1 nef protein was reported to downmodulate NKG2D ligands and inhibit NK cell cytotoxicity [77]. Soluble ligands for the NKG2D are released during HIV-1 infection and impair NKG2D expression and cytotoxicity of NK cells suggesting that HIV-1 may indirectly suppress NKG2D-mediated NK cell responses [74]. NKG2D binds to cytomegalovirus glycoprotein UL16 and activates NK cell cytotoxicity [78]. Ligation through NKG2D on NK cells results in the lysis of microglial cells suggesting a role of NK cells in the CNS [79]. Disruption of the NKG2D-NKG2D ligand interaction using blocking antibodies significantly inhibited NK mediated killing of primary human oligodendrocytes [73]. Downregulation of NKG2D caused by tumor-derived TGF-β has been reported in patients with glioblastoma leading to immune escape of the tumor [35, 80].

NKp46

NKp46 is expressed on resting and activated NK cells but not on T cells or B cells. Expression of NKp46 is higher in non-AIDS vs. AIDS patients and the level of NKp46 expression directly correlated with the degree of NK cell cytotoxicity [81]. Binding of NKp46 to

haemagglutinin of influenza virus activates their lysis by NK cells [82]. Blocking of NKp46 prevented the killing of microglia by NK cells, suggesting that NK cells may be able to restrict the immune response in the CNS [79]. HIV-1 infection decreases the expression of NKp46 and overall decreases NK cell activation [83, 84].

CD38

CD38 is expressed on NK cells and astrocytes, as well as T cells, B cells, monocytes, platelets, and erythrocytes [85, 86]. The natural ligand for CD38 is CD31, however, recent studies discovered a novel ligand for CD38 on dendritic cells that is not CD31 [87]. In astrocytes, CD38 functions to regulate calcium signaling and pro-inflammatory cytokine and chemokine production [31, 88]. Expression of CD38 is upregulated in HIV-1 activated astrocytes and in HAND brains [88]. Astrocyte CD38 levels are upregulated by interleukin (IL)-1β, and this effect is further amplified by HIV-1 envelope glycoprotein (gp120) leading to eventual neuronal damage [31, 32, 88].

Signaling through CD38 on NK cells induces cytotoxicity against mastocytoma cells [89] and the release of IFN-γ [90]. NK cell cytotoxicity against astrocytes via CD38 has not yet been investigated. In HIV-1-infected patients, increased T cell CD38 expression indicates disease progression, whereas decreased CD38 expression is a good indicator of the effectiveness of anti-retroviral therapy [91]. Thus CD38 plays an important role in HIV-1 infection.

1.6 NK Cells & HIV-1

NK cells undoubtedly play a role in the immune response against HIV-1. NK cells can limit HIV replication through direct killing of infected cells as well as the secretion of anti-viral

cytokines and chemokines that suppress HIV-1 replication [83, 92]. One study demonstrated that NK cells suppression of HIV-1 is mediated predominantly by secretion of CC-chemokines MIP-1α, MIP-1β, and RANTES [92, 93]. The presence of viremia from HIV-1 drastically impaired NK cells' inhibitory function on HIV replication [92, 93]. NK cells from HIV patients show a functional impairment to kill tumor cells, a possible explanation for the increase in opportunistic tumors in HIV patients [84]. HIV-1 exposed but not infected individuals showed an increase in NK cell function suggesting a protective effect [6, 94]. Conversely, HIV decreases the expression of NCRs, overall decreasing NK cell activation [83, 84]. Expression of NK activating receptor KIR3DS1 in combination with HLA-B allele is associated with delayed progression to AIDS and KIR3DS1 in the absence of HLA-B allele is associated with more rapid progression to AIDS [95]. Not only is NK cell receptor expression altered during HIV-1, their ligand expression can also be altered. HIV induces the NKG2D ligands and downregulates CD48 ligand [53].

1.8 NK Cells in the CNS

Following traumatic, infectious, and autoimmune-mediated brain injury, NK cells have been found in the CNS, but the functional significance of NK cell recruitment and their mechanisms of action during brain inflammation are not well understood [35]. NK cell function in the CNS following brain injury could either be neuroprotective or neurotoxic (Figure 1.4) [35]. Some evidence suggests that in the context of experimental autoimmune encephalomyelitis (EAE), NK cell activity suppresses CNS inflammation by their ability to kill proinflammatory microglia which reduces the neurotoxicity of autoreactive T cells [35, 79] and NK cell IFN-γ production could lead to neuroprotection through the activation of microglia [35]. During multiple sclerosis, NK cells can initiate neurotoxicity by killing oligodendricytes [73].

Alternatively, in cerebral malaria, NK cell IFN-γ production activates cytotoxic T cells and dendrictic cells leading to neurotoxicity [35, 96].

Although immunoregulatory function of NK cells on neurological diseases and CNS infection remains understudied, several studies show NK cell modifications associated with neurological disorders such as multiple sclerosis [97], glioblastoma [80], HSV1 encephalitis [98], and Alzheimer's [99, 100]. Downregulation of NK activating receptor NKG2D caused by TGF-β was reported in glioblastoma patients and this downregulation lead to immune escape of the tumor [35, 80]. Recent studies observed an increase in 5-HT(2C) receptor for serotonin on NK cells from Alzheimer's patients (31). The over expression of 5-HT (2C) lead to decreased NK cell activity [35, 100]. Patients with Parkinson's disease show diminished NK cell activity [101]. In a postmortem study on multiple sclerosis patients, NKp46+ NK cells were found in brain lesions [35, 102]. NK cells have also been reported to kill oligodendrocytes, astrocytes and microglia cells[54, 79]. These studies suggest that brain-infiltrating NK cells play a role in immune responses in the CNS via their interaction with CNS resident cells.

Even though NK cells in the CNS are understudied, it is evident that NK cells can participate in immune responses in the brain. Monitoring NK cell receptors and ligands following brain injury as markers for disease activity, as well as understanding the mechanism of recruitment and the function of brain-infiltrating NK cells following during brain injury could lead to the development of new novel NK cell based therapies for brain disorders.

NK Cells and Astrocytes

Very few studies have investigated NK cell interactions with astrocytes. Previous studies reported NK cells are capable of killing astrocytes and that IL-2 augments NK cell killing of

astrocytes [54, 73, 103]. Our study aims to identify important molecules involved in NK cell signaling on astrocytes that confer this NK directed cytotoxicity of astrocytes.

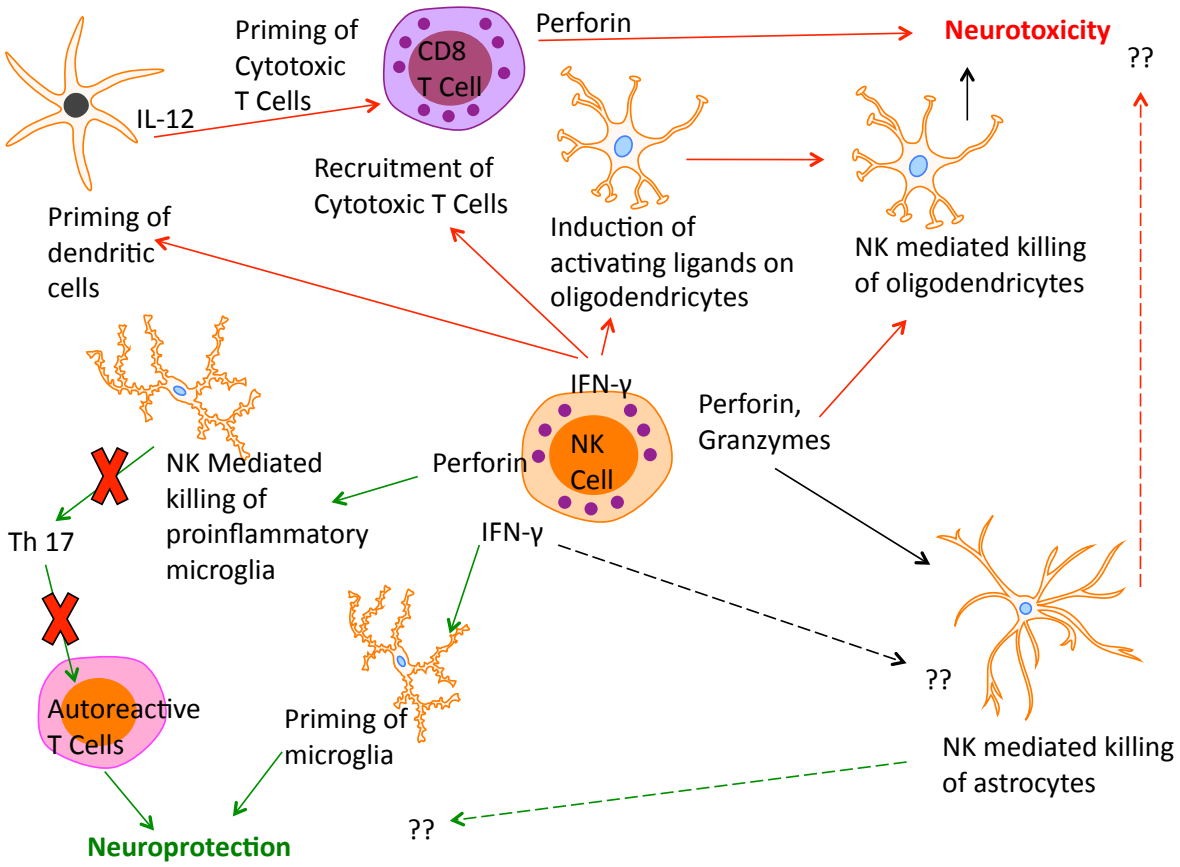

Figure 1.4 NK cell activity in the CNS could either lead to neuroprotection or neurotoxicity. In cerebral malaria models, secretion of IFN-γ by NK cells lead to activation of cytotoxic T cells and dendritic cells resulting in diminished animal survival. Cytotoxic perforin and granzymes granule may also initiate inflammatory cascades leading to increased brain injury. Alternatively, IFN-γ could generate neuroprotection through activation of microglia. In the context of EAE, NK cells reduce neurotoxicity by killing proinflammatory microglia. Alternatively, NK cells could initiate neurotoxicity by killing oligodendricytes. NK cells were reported to kill astrocytes but their mechanism of action and effect on astrocytes has not previously been investigated.

1.9 Significance

Although the use of highly active antiretroviral therapy (HAART) has led to a strong reduction of HAD incidence, the prevalence of HIV-1 associated cognitive impairment still remains a common problem in AIDS patients [10]. Astrocytes are now known to be important during the pathogenesis of HAND, but their assessment, pathogenesis, and treatment of HIV-1 in the CNS remain elusive. NK cells can infiltrate into the CNS during HIV-1 infection. The interactions between NK cells and CNS resident cells are understudied and there is no previous data on NK cell effects on HIV-1 infected astrocytes. Considering the importance of astrocytes for the function of the CNS, there is an urgent need to advance the study of HIV-1 infection of astrocytes. Studies providing novel insight into the role of NK cells in the CNS and their interactions with astrocytes will not only expand our knowledge of the roles NK cells play in the CNS, but could also suggest for the development of NK cell-based approaches to treat HAND patients.

The ***goal*** of this study aimed to identify what ligands astrocytes express that allow for NK cell interaction and aimed to determine the functional significance of NK cell -astrocyte interactions in the context of HIV-1 infection of the CNS. We ***hypothesize*** that HIV-1 infection of the CNS alters the expression of NK cell ligands on astrocytes leading to destruction of astrocytes by NK cell mediated cytotoxicity.

The following ***specific aims*** were used to test our hypothesis:

Specific Aim 1: To determine the expression of receptor-ligands involved in NK cell-astrocyte interactions in the presence and absence of HIV 3S peptide.

Specific Aim 2: To determine the functional significance of receptor-ligands involved in NK cell-astrocyte interactions in the presence and absence of HIV 3S peptide.

CHAPTER 2

MATERIALS AND METHODS

2.1 Isolation and Cultivation of Primary Human Cells

Primary human fetal astrocytes: Primary human fetal astrocytes were kindly donated to us from Anuja Ghorpade's lab, University of North Texas Health Science Center (Fort Worth, Texas). From their methods [104]: Human astrocytes were isolated from first- and early second-trimester aborted specimens, ranging from 82 to 127 days, obtained from the Birth Defects Laboratory, University of Washington, Seattle, WA in full compliance with the ethical guidelines of the NIH. The institutional review boards of both the Universities of Washington and North Texas Health Science Center approved the collection of human tissues for research. The Birth Defects Laboratory obtained written consent from all tissue donors. Brain tissues were dissected and mechanically dissociated. Cell suspensions were centrifuged, suspended in media, and plated at a density of 20×10^6 cells/150 cm2. The adherent astrocytes were treated with 0.25% trypsin-EDTA (ethylenediaminetetraacetic acid) (Life Technologies, Carlsbad, CA) and cultured under similar conditions to enhance the purity of replicating astroglial cells (101). The astrocyte preparations were routinely >99% pure as measured by immunocytochemistry staining for glial fibrillary acidic protein and microglial marker CD68 determine possible microglial contamination and contribution of microglia in inflammatory responses [104]. Maintenance of astrocytes took place in DMEM/F12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) (Life Technologies, Carlsbad, CA) supplemented with 1mM sodium bicarbonate, 10% fetal

bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and 10mM antibiotic-antimycotic (Life Technologies, Carlsbad, CA) at 37°C in a humidified 5% CO2/95% air environment.

Primary human NK cells: Whole blood was taken from healthy volunteers with prior approval from UNTHSC IRB. Peripheral blood mononuclear cells (PBMCs) were purified by ficoll separation using a histopaque gradient. Red blood cells were lysed with 5 mls of ACK (0.15M ammonium chloride, 10mM Potassium bicarbonate, 0.1mM disodium EDTA) followed by the addition of 20 mls of phosphate buffered saline (PBS) + EDTA to stop the lysing reaction. Primary NK cells were then isolated from PMBCs by negative selection using a MACS NK isolation kit (Miltenyi Biotec, Cologne, Germany). Maintenance and activation of primary NK cells took place in RPMI 1640 supplemented with 1mM sodium bicarbonate, 10% FBS,10 mM HEPES, 10 mM non essential amino acids, 10 mM sodium pyruvate, and 1000 U/ml human interleukin-2 (IL-2) (eBioscience, San Diego, CA) at 37°C in a humidified 5% CO2/95% air environment for 1 week prior to use.

2.2 Maintenance of Cell Lines

HEK-293 and Jurkat cells: HEK-293 and Jurkat cells were maintained RPMI 1640 supplemented with 1mM sodium bicarbonate, 10% FBS, 10 mM HEPES, 10 mM non essential amino acids, 10 mM sodium pyruvate, and 10mM antibiotic-antimycotic.

DB cells: DB cells were maintained in RPMI 1640 supplemented with 10% FetalPlex (Gemini Bio-Products, West Sacramento, CA), 2 mM glutamine, 10 mM HEPES, 10 mM sodium pyruvate, 10 mM non essential amino acids, and 10mM antibiotic-antimycotic.

NK92 cells: NK92 cells were grown and cultivated in minimum essential medium (MEM) alpha medium (Life Technologies, Carlsbad, CA) supplemented with 12.5% FBS and donor horse serum (Atlanta Biologicals, Lawrenceville, GA), 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, and 0.02 mM folic acid.

2.3 HIV-3S Peptide Stimulation

To determine if HIV 3S peptide modulated the expression of receptors and ligands, cells were resuspended at 1×10^5 cells/500 µl in Opti-MEM® I reduced serum medium (Life Technologies, Carlsbad, CA) plated in a 96 well round bottom plate with increasing concentrations of the HIV peptide Ac-SWSNKS-Nh2 (3S) (Celtek Peptides, Nashville, TN) at 1 µg/ml and 10 µg/ml added to different wells. The incubation period was 4 hours and took place at 37°C in a humidified 5% CO2/95% air environment. After incubation astrocytes were washed with PBS and detached with 0.25% trypsin-EDTA.

2.4 Production of NKp44 Fusion Protein

The expression of NKp44L was established utilizing a NKp44 fusion protein. The recombinant fusion protein is a dimmer containing two extracellular domains of NKp44 in the arm region of human IgG1, thus maintaining the immunoglobulin domain structure of NKp44 extracellular domain (Figure 2.1 A). In order to make the NKp44-Fc fusion protein, we subcloned the V and C2 domain of the NKp44 into pCD5lneg1 vector which contains the CH2 and CH3 regions of human IgG1 (Figure 2.1 B). The extracellular domain of the NKp44 was amplified by PCR (forward primer NKp44NheIFP-5′TCGCTAGCGCAATCCAAGGCTCAGGT-3′ and reverse primer NKp44BamHIRP-5′ CTCGGGATCCGTGTCTGCAGG GCCA-3′). The amplified

products were digested with NheI and BamHI and cloned into the NheI-BamHI cut pCD5lneg1 mammalian expression vector. To produce soluble NKp44 fusion protein, the NKp44 plasmid was transiently transfected into HEK-293 cells using Fugene-6 transfection reagent (Roche Diagnostic corporation, Indianapolis, IN) at a ratio of 3 µl Fugene to 1 µg DNA. Transfected cells incubated in Opti-MEM with replacement of media every 24 hours and collection of supernatants on day 2. Following collection, supernantants were centrifuged to remove cellular debris, and then concentrated to 1 µg with a 35,000 molecular weight centrifugal concentrator, Amicon Ultra 10k (Millipore, Billerica, Ma). Analysis of the fusion protein for functionality was done via western blot and by using flow cytometry. Binding of the fusion protein was tested on DB cells, a diffuse large cell lymphoma cells, known to express the ligand for NKp44.

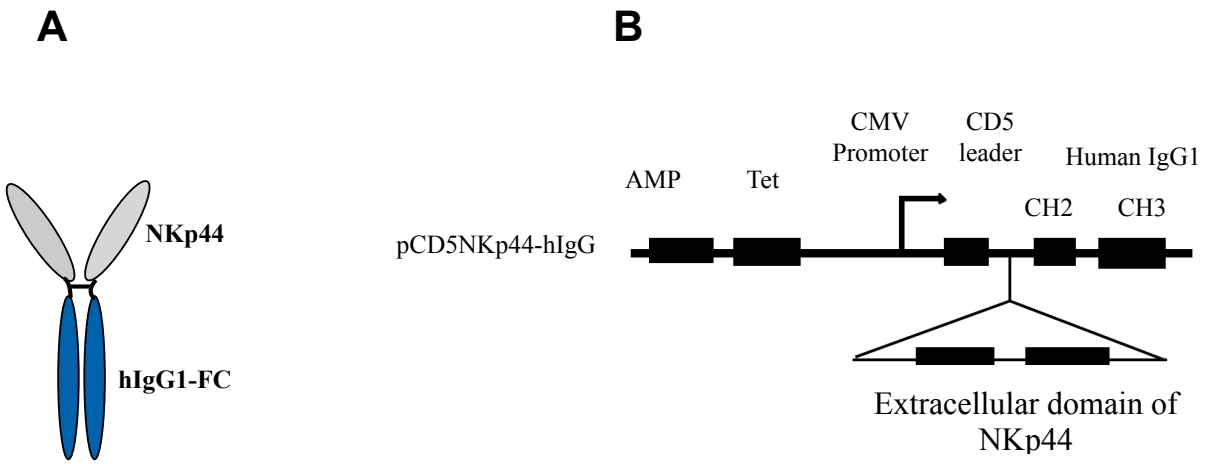

Figure 2.1 Characterization of NKp44-Fc fusion protein. Schematic representation of NKp44 fusion protein (A) and the mammalian expression vector construct (B). The region corresponding to the extracellular Ig V and C2 domains of NKp44 was PCR amplified and subcloned in front of the CH2, CH3 domain of human IgG1 inframe with the CD5 leader sequence and CH2.

2.5 Flow Cytometry

Determination of the expression of CS1, 2B4, CD48, and LLT1 on astrocytes: Astrocytes treated with 10 µl/ml HIV-1 3S peptide or astrocytes that received no HIV-1 3S peptide stimulation were resuspended in 500 µl PBS-BSA buffer (PBS, 1% bovine serum albumin) and first incubated with Human IgG Fc fragment (Rockland, Gilbertsville, PA) to block nonspecific interactions with Fc receptors. Cells were resuspended such that each different treatment or control group had 1×10^6 cells/500 µl PBS-BSA. Fc blocked cells incubated for 30 minutes on ice with 2.5 µg of one of the following monoclonal antibodies conjugated to phycoerythrin (PE) (Biolegend ,San Diego, CA: anti-human CD319 (CRACC,CS1), anti-human CD244 (2B4), anti-human CD48, or anti-human CD161 (LLT1). As negative controls, cells incubated for 30 minutes with 2.5 µg of PE mouse IgG2b isotype for CRACC, 2.5 µg of PE mouse IgG1 isotype for anti-CD244, anti-CD48, and anti-CD16. Cells were then washed two times and resuspended in 500 µl buffer. Cells expression was analyzed using a Beckman Coulter Cytomics FC500 flow cytometry via FL-2 for the PE conjugated antibodies. The mean fluorescent intensity (MFI) was quantified for each anti-body astrocytes were stained with. This value was subtracted from the MFI of the isotype control.

Determination of the expression of NKG2D, NKp44, NKp46, and CD38 on NK92 cells and primary human NK cells after incubation with astrocytes: Astrocytes and either NK92 cells or primary NK cells were incubated at a 2:1 ratio in a 96 well round bottom plate such that each corresponding well received 2×10^5 NK cells in 100ul of their media and 1×10^5 astrocytes in 100 µl of their media. Astrocytes and NK cells incubated together for 4 hours or overnight at 37°C in a humidified 5% CO2/95% air environment. Cells were washed with PBS, treated with 0.25% trypsin-EDTA to detach the astrocytes, Fc blocked, and resuspended in PBS-BSA so that

each treatment and control group had approximately 1×10^6 cells. For the determination of CD38 expression, the cells were stained with 2.5 µg Alexa Fluor® 488 anti-human CD38 antibody for 30 minutes followed by 2 washes with buffer and then stained with 2.5 µg anti-human 2B4 PE for 30 minutes followed by 2 washes. To separate the NK cells, 2B4+ cells will be gated on using the FL-2 channel. CD38 expression will be analyzed using FL-1 channel. NK cells without astrocyte incubation will be stained with 2.5 µg Alexa Fluor® 488 anti-human CD38 antibody and with 2.5 µg anti-human 2B4 for 30 minutes and then will be analyzed by flow cytometry to use as comparison. NK cells with and without astrocyte incubation were incubated with only 2.5 µg PE and FITC mouse IgG1 isotype to use as negative controls. For the determination of NKG2D, NKp44, and NKp46 astrocytes were treated the same as above except were stained with 2.5 µg PE anti-human NKG2D, 2.5 µg PE anti-human NKp44, and 2.5 µg PE anti-human NKp46 antibodies instead of Alexa Fluor® 488 anti-human CD38 antibody and with FITC anti-human 2B4 antibody instead of PE anti-human 2B4. These NK cells were separated by gated on 2B4+ population using FL-1 channel. NK cells with and without astrocyte incubation were incubated with only 2.5 µg PE and FITC mouse IgG1 isotype to use as negative controls. Astrocytes express CD56+. Our preliminary studies showed that astrocytes little 2B4. Analysis of cell surface receptor expression was done via flow cytometry. MFIs were calculated as previously described in 5. NK92 and primary NK cells were stimulated with 10 µl/ml HIV 3S peptide for 4 hours and CD38 expression of peptide stimulated NK cells in the presence and absence of astrocytes was analyzed.

Determination of induction of NKp44L on astrocytes with HIV-1 3S peptide stimulation:

To test whether HIV 3S peptide induced the expression of NKp44L , 70 µg of NKp44-Ig fusion protein was added to 1×10^6 FC blocked astrocytes and Jurkat lymphocyte cells that received

1μg/ml HIV-1 3S peptide , 10 μl/ml HIV-1 3S peptide, and cells that received no peptide stimulation. Astrocytes and Jurkat cells incubated with the fusion protein on ice for 45 minutes prior to incubation with the secondary antibody. After fusion protein incubation, cells were washed 2 times with buffer and then incubated with 2.5 μg of the secondary antibody, anti-hIgG-Fc-PE (Biolegend ,San Diego, CA) for 30 minutes and analyzed via FL-2. Cells just receiving the secondary antibody with no fusion protein incubation were used as negative controls. MFIs were calculated as previously described in 5.

2.6 Cytotoxicity Assay

The killing of astrocytes by blocking NKp44 interactions or CD38 interactions on NK cells was tested using a Chromium-51 radionucleotide (^{51}Cr) release assay. Killing assays were done on astrocytes with 10 μg/ml HIV 3S and astrocytes with no peptide stimulation using NK92 cells or primary NK cells as the effector cells. Astrocytes and NK cells were incubated with human IgG Fc fragment to block interactions with Fc receptors on both cells prior to use. Cells were resuspended in their corresponding media. Astrocytes were incubated with ^{51}Cr (PerkinElmer, Waltham, MA) at a concentration of 1/5 ^{51}Cr to 4/5 medium for 1 hour 37°C in a humidified 5% CO2/95% air environment. Astrocytes were washed three times with 1ml media and resuspended in media such that the same amount of astrocytes was distributed in 100μl of media for each well of a standard 96 well round bottom plate. ^{51}Cr labeled astrocytes were then treated with 15 μg of NKp44 fusion protein , 1 μg anti-CD38 or 1 μg mIgG1 isotype. NK cells were tested by flow cytometry for the expression of NKp44 by a 30 minute incubation with anti-NKp44 PE (Biolegend, San Diego, CA) prior to use on killing assays involving the blocking of NKp44 interactions. To block NKp44 and CD38 on NK cells, NK cells were incubated with 2.5 μg anti-

CD38 or anti-NKp44 or mIgG1 isotype. The effector NK cells were serially diluted such that there were triplicate wells with effector to target ratios of 20:1, 10:1 and 5:1. Wells were made using HIV 3S peptide treated and untreated target cells in medium only with no anti-body blocking treatment to get a background release of chromium and wells were made with HIV 3S peptide treated and untreated target cells without blocking in nonidet P-40 lysis buffer to give the measurement of the 100% lysis. After the effector and target cells were distributed into the plates, they incubated together for 4 hours at 37˚C, cells were spun down and 100 ul of the supernatant was collected and added to 2ml scintillation cocktail fluid in a standard scintillation vial. Samples ran on a Perkin Elmer scintillation counter. Percent specific lysis was calculated using the equation:

[(Mean cpm in experimental sample)- (mean cpm of spontaneous release) / (mean cpm of maximal release) - (mean cpm of spontaneous release)] X 100.

2.7 Enzyme-Linked Immunosorbent Assay (ELISA)

Quantification of IFN-γ production from astrocyte-NK cell co-culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA) (eBioscience, San Diego, CA). NK92 cells and astrocytes were first Fc blocked . NK92 cells were then treated with 2.5 µg anti-NKp44 or anti-CD38. NK92 cells with no antibody or with 2.5 µg mIgG1 were used as controls. Astrocytes with and without HIV 3S peptide stimulation were treated with 1 µg anti-CD38, mIgG1 isotype control or received no antibody treatment. NK92 cells and astrocytes incubated overnight in a 96 well plate such that the ratio of NK cells to astrocytes was 10:1. The supernatants were collected and plated for and ELISA. 96 well ELISA plates were coated with 100 µl/well capture antibody and incubated overnight at 4° C. The wells were washed 5 times

with 250 µl wash buffer (1XPBS, 0.05% tween). Wells were blocked with 200 µl assay diluent at room temperature for 1 hour and then washed five times. Next, 100 µl of the standards and samples diluted in assay diluent were then added into the appropriate wells and the plate incubated overnight at 4° C for maximal sensitivity. Next, the plate was washed 5 times with 250 µl/well wash buffers and 100 µl biotin-conjugate was added to each well. Plates were then covered and incubated at room temperature for 1 hour. Following incubation, plates were washed 5 times with 250 µl wash buffer per well. 100 µl of diluted avidin-HRP was then added to all wells and plates were incubated for 30 minutes at room temperature. Following incubation, plates were washed 7 times as before and 100 µl TMB substrate solution was added to all wells. Plates were then covered and incubated at room temperature for 15 minutes. Subsequently, 100 µl of stop solution (1 M H_3PO_4) was added to each well and plates were immediately read at an absorbance of 450nm using a microplate reader (Dynex Technologies, Inc, Chantilly, VA). IFN-γ levels were determined by comparison with standard curves determined by 5-parameter curve fits. Both NK cells and astrocytes can release IFN-γ. IFN-γ release of astrocytes and NK cells cultured alone with 1 µg anti-CD38, 1 µg anti-NKp44, mIgG1 isotype control or no antibody treatment was quantified and these values were subtracted from the IFN-γ release of astrocytes and NK cells co-cultured together.

2.8 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6.0 software (San Diego, CA) using student's t –test or ANOVA with multiple comparisons using Tukey's correction. A p value <0.05 was considered significant. Each astrocyte experiment will be performed with a minimum of three independent donors unless stated otherwise. Plotted data is displayed as means with standard error of the mean (SEM).

CHAPTER 3

CELL SURFACE EXPRESSION OF IMMUNE RECEPTORS ON NK CELLS AND THEIR LIGANDS ON ASTROCYTES AND THE EFFECT OF HIV 3S PEPTIDE ON THEIR EXPRESSION

3.1 Introduction

HIV-1 infection of astrocytes disrupts the integrity of the BBB [34], allowing for NK cells to infiltrate in the CNS [35-37]. NK cells are important in immunity against HIV-1 infection and can control viral infections by releasing IFN-γ or killing virally infected cells [42, 44]. Their functions are regulated by inhibitory and activating receptors binding corresponding ligands on the surface of target cells [42, 45, 47]. There is little data on ligands expressed on astrocytes that could allow for interaction with NK cells and impact NK cell function and the role of NK cells. NK cell interactions with HIV-1 infected astrocytes have not previously been evaluated. To address a possible immunoregulatory function of NK cells on astrocytes infected with HIV-1, we first evaluated the surface expression of receptors and ligands that could allow for NK cells astrocytes to interact and assayed their expression level in the presence and absence of HIV 3S peptide.

We first examined astrocytes for the cell surface expression of receptors and ligands important during NK cell signaling: 2B4, CD48, CS1, and LLT1. Our lab previously cloned 2B4, CS1, and LLT1. Studies show these receptors and ligands differentially regulate the function of cells. Previous studies in our lab suggest $2B4^+$ $CD8^+$ T cells play an inhibitory role against constrained HIV epitopes [50]. CD48, the high affinity ligand for 2B4, is found to be

downregulated during HIV-1 infection [53]. CS1 induces cytokine production in B cells [59]. In contrast, in monocytes, CS1 inhibits the production of cytokines [60]. LLT1 expression is upregulated on glioblastoma cells [67] and LLT1 expression was detected on B cells following *in vitro* infection with EBV and HIV-1 [63]. Thus, these receptors play important roles in viral infections and their expression can be altered during the course of HIV infection [53].

In this study, 2B4, CD48, LLT1, and CS1 expression on astrocytes was evaluated in the presence and absence of HIV 3S peptide to determine if HIV-1 alters their surface expression on astrocytes. Furthermore, astrocytes may secrete cytokines that affect expression of receptors on NK cells important in NK cell signaling. Therefore, we investigated if interaction with astrocytes affects the expression levels of CD38, NKG2D, NKp44, and NKp46 on NK cells. CD38 is expressed on both NK cells and astrocytes [85, 86] and has an important implication in HIV-1 and HAND. Expression of CD38 was previously reported to be upregulated in HIV-1 activated astrocytes by gp120 and in HAND brains [88]. We evaluated if HIV-1 gp41 3S peptide upregulates CD38 expression on activated NK cells.

The ligand for NKp44 also has an important implication in HIV-1. NKp44L has not yet been detected on circulating leukocytes isolated from healthy individuals, but it is expressed on a large panel of the tumor and transformed cells. In previous studies using uninfected T cells, HIV 3S peptide induced expression of NKp44L. Furthermore, increasing concentrations of HIV 3S peptide increased NKp44L expression on T cells resulting in their lysis [6]. We examined if HIV 3S peptide induced NKp44L expression on astrocytes and compared the expression to Jurkat T cells. The results from this study will enhance our understanding of how NK cells and astrocytes can interact in the context of HAND.

3.2 Results

Astrocytes did not express significant levels of 2B4, CD48, CS1 or LLT1

Surface receptor proteins are a vital part in the way cells interact with other cells. Cell surface expression of 2B4, CD48, CS1, and LLT1 on astrocytes was analyzed via flow cytometry. MFIs were calculated and subtracted from the control MFIs. Astrocytes positive for the sample expression will have a MFI value that is greater than the mean for the control and is statistically significant. Astrocytes examined did not express 2B4, CD48, CS1 or LLT1 (Figure 3.1). Although some astrocyte donors expressed low levels of CS1 and LLT1, the expression was not statistically significant as compared to the control (Figure 3.1).

HIV 3S peptide did not induce the expression of 2B4, CD48, CS1 or LLT1 on astrocytes

To determine if HIV-1 modulates receptor expression, astrocytes were stimulated with 10 μg/ml HIV-1 3S peptide for 4 hours prior to being stained with anti-2B4 antibody, anti-CD48 antibody, anti-CS1 antibody and anti-LLT1 antibody. Expression was analyzed using flow cytometry. Astrocytes stimulated with HIV 3S peptide and incubated with mIgG1 isotype control were used as negative isotype controls. HIV 3S peptide stimulation had no effect on the expression of 2B4, CS1, and LLT1 (Figure 3.2 A, C, D). Stimulation with HIV 3S peptide seemed to further downregulate the expression of CD48, but the decrease was not statistically significant as compared to the control (Figure 3.2 B).

Interaction with astrocytes decreases the expression of CD38 on NK92 cells

Astrocytes can secrete a battery of cytokines that may alter NK cell receptor expression. To determine the effects astrocytes have on NK92 cell receptor expression, NK92 cells were incubated with astrocytes at a ratio of 2:1 for either 4 hours or overnight. Expression of NKG2D, NKp44, NKp46, and CD38 was analyzed by flow cytometry. MFIs of NK92 cells in the presence

of astrocytes were compared to the MFIs of NK92 cells that had no astrocyte interaction. CD56 is a normal marker used to gate on NK cells, however, astrocytes express CD56 as well. Since our studies showed astrocytes do not express 2B4 (Figure 3.1) and NK cell 2B4 expression remained the same even after NK cells interacted with astrocytes (Figure 3.3 A), 2B4 was used to gate on NK cells (Figure 3.3 B). NKp44, NKp46, and NKG2D expression did not change on NK92 cells after 4 hour (Figure 3.4 A) and overnight (Figure 3.4 B) incubation with astrocytes. NK92 interaction with astrocytes significantly decreased CD38 expression on NK92 cells after 4 hours (Figure 3.4 A). CD38 expression was slightly decreased after overnight incubation with astrocytes, however, overnight incubations resulted in a large population of dead cells and this decrease was not statistically significant (Figure 3.4 B).

Interaction with astrocytes increases the expression of CD38 on primary NK cells.

To determine the effect astrocytes have on primary NK cell receptor expression, the experiments were conducted under the same specifications as for NK92 cells stated above. Co-culture with astrocytes significantly increased CD38 expression on primary NK cells at the 4 hour time period (Figure 3.5 A). CD38 expression was also increased after overnight incubation but, the increase was not significant (figure 3.5 B). There were no substantial changes in NK2GD, NKp44 or NKp46 expression on primary NK cells after 4 hour (Figure 3.5 A) or overnight (Figure 3.5 B) co-cultures with astrocytes.

CD38 expression on NK cells does not increase with HIV 3 S peptide stimulation.

To investigate if HIV-1 the conserved 3S peptide of the HIV-1 envelope protein gp41 increases expression on NK cells after interaction with astrocytes, NK92 cells (Figure 3.6 B) and primary NK cells (Figure 3.6 C) cells were co-cultured with astrocytes at a ratio of 2:1 in the presence and absence of HIV 3S peptide. CD38 surface expression was analyzed via flow

cytometry. NK cells were gated on using 2B4 (Figure 3.6 A). MFIs were compared to cells without HIV 3S peptide stimulation and mIgG1 was used as an isotype control. HIV 3S peptide had no effect on the expression of CD38 on NK92 cells or primary NK cells cultured alone or co-cultured with astrocytes (Figure 3.6 B,C).

NKp44L is expressed on astrocytes and HIV 3S peptide downregulates its expression

We studied the expression and function of NCR NKp44 on NK- astrocytes interactions in the presence or absence of an HIV 3S peptide using a fusion protein consisting of the extracellular domain of NKp44 fused to Fc portion of human IgG. HIV 3S peptide was shown induce NKp44L on T cells and increasing concentrations of HIV 3S peptide increased NKp44L expression [6]. Our lab replicated these studies using Jurkat T cells and achieved similar results (Figure 3.7). NKp44L expression was induced with HIV 3S peptide stimulation at 1 μg/ml and further increasing the peptide concentration to 10 μg/ml further increased NKp44L expression (Figure 3.7). We repeated the studies using astrocytes and determined that astrocytes significantly express NKp44L (Figure 3.8). Incubation of astrocytes with increasing concentrations 3S peptide downregulated NKp44L expression on astrocytes (Figure 3.8 A,B). The decrease in expression of NKp44L with increasing HIV 3S peptide concentrations was not statistically significant as compared to NKp44L expression in the absence of HIV 3S peptide.

3.3 Conclusions

The astrocytes that were examined did not express high levels of 2B4, CD48, CS1, and LLT1. Stimulation with HIV 3S peptide did not change the expression level of 2B4, CS1, or LLT1; however, stimulation with HIV 3S peptide further downregulated expression of CD48,

although, the decrease is not statistically significant as compared to the control [18]. CD48 is reported to be downregulated on T cells during HIV-1 infection [53].

Astrocytes may secrete cytokines that could alter NK cell receptor expression [18]. Astrocytes had no effect on NKG2D, NKp44 and NKp46 expression on NK92 cells and primary NK cells. CD38 was downregulated on NK92 cells after interaction with astrocytes. In contrast, CD38 was upregulated on primary NK cells after interaction with astrocytes. Overnight incubations resulted in too many dead cells. From this data we cannot conclude if the astrocytes are secreting cytokines that alter expression on NK cells or if the alteration of CD38 expression is from cell-cell contact. The induction of receptors and increase in expression of receptors may take longer than 4 hours. To assess dependency on soluble factors as opposed to direct cell-cell contact future studies can repeat this experiment again quantifying CD38 expression on NK cells in Transwells vs. non-Transwell co-cultures with astrocytes. Expression of CD38 is upregulated in HIV-1 activated astrocytes by gp120 and in HAND brains [88]. When stimulated with HIV-1 gp41 3S peptide, we saw no change in expression on NK cells even after with astrocytes. Thus, CD38 has an important implication in NK-astrocytes interactions.

HIV 3S peptide was shown to induce NKp44L on T cells and increasing concentrations of HIV 3S peptide increased NKp44L expression. On astrocytes NKp44L was significantly expressed even without HIV 3S peptide stimulation. This is the first report of NKp44L on healthy cells [70, 71]. Interestingly, increasing the peptide concentration on astrocytes decreased NKp44L expression. Thus, HIV-1 downregulating NKp44L expression on astrocytes predicts a protective effect of astrocytes from NK cell functions.

The first aim of the study was exploratory and aimed to identify NK associated molecules on NK cells and astrocytes that allow for astrocytes and NK cells to interact. We reported an

alteration of CD38 expression on NK cells after incubation with astrocytes and expression of a novel ligand for NKp44 on astrocytes. The results from this aim confer that astrocytes express ligands that allow for NK cell interaction. The second aim of this study investigated the function of NKp44L on astrocytes and CD38 in regards to NK cell-astrocyte interactions in the presence and absence of HIV 3S peptide stimulation.

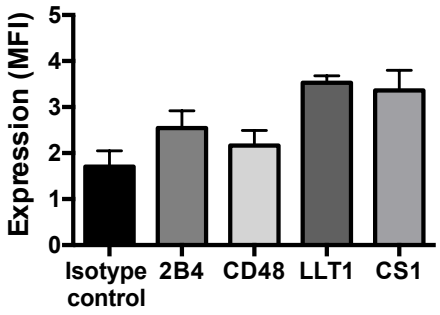

Figure 3.1 Surface expression of ligands and receptors important in NK cell signaling on astrocytes. Astrocytes were incubated with 2.5 µg of one of the following antibodies: 2B4, CD48, CS1, and LLT1 and expression was analyzed by flow cytometry. Astrocytes incubated with 2.5 µg mIgG were used as negative isotype controls. Mean Fluorescent Intensity (MFI) values, the differences between the mean value for the control group and the experimental groups were quantified. Figure is representative of combined MFIs of 3 independent experiments done in duplicate. Results are expressed as mean ± SEM, student's t-test

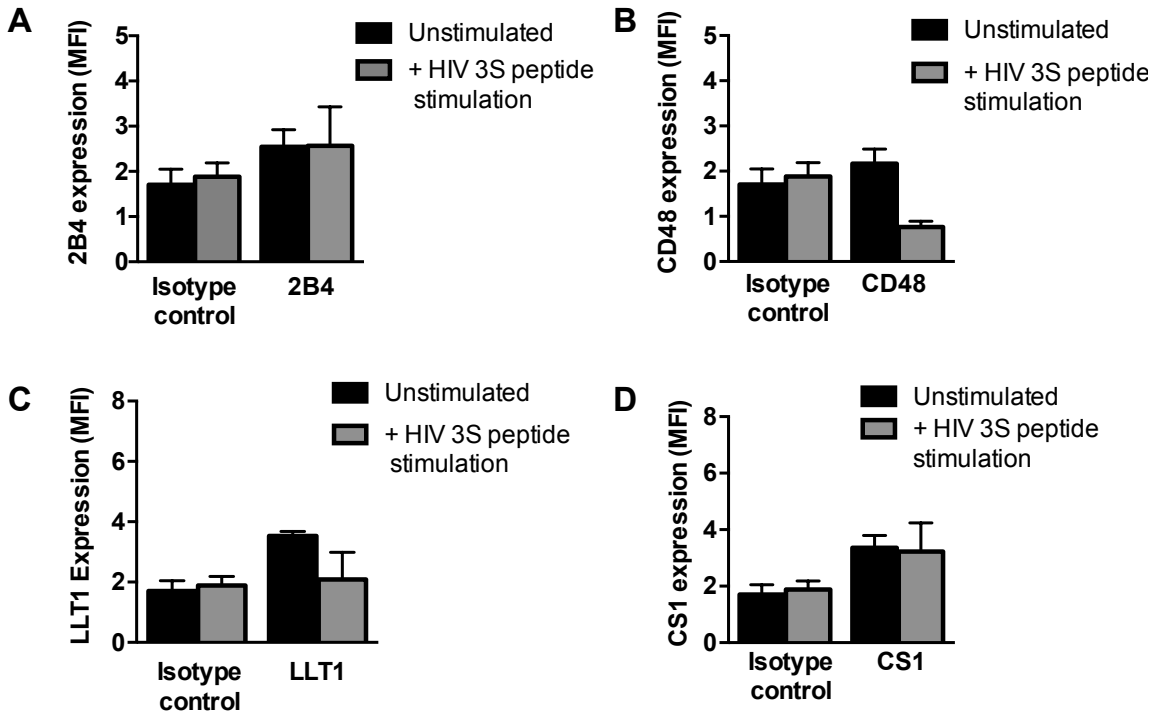

Figure 3.2 HIV 3S peptide did not induce the expression of 2B4, CD48, CS1 or LLT1 on astrocytes. Astrocytes were incubated with 10 µg/ml HIV 3S peptide for 4 hours prior to staining with 2.5 µg of one of the following antibodies: 2B4 (A), CD48 (B), CS1 (C), LLT1(D). Astrocytes incubated with mIgG1(A-C) and mIgG2b (D) were used as a negative isotype controls. To calculate the MFI, the mean value for the control was subtracted from the mean value for each group. MFIs were compared with (grey bar) and without (black bar) HIV 3S peptide stimulation. Figure is representative of 3 independent experiments done in duplicate. Results are expressed as mean ± SEM, student's t-test

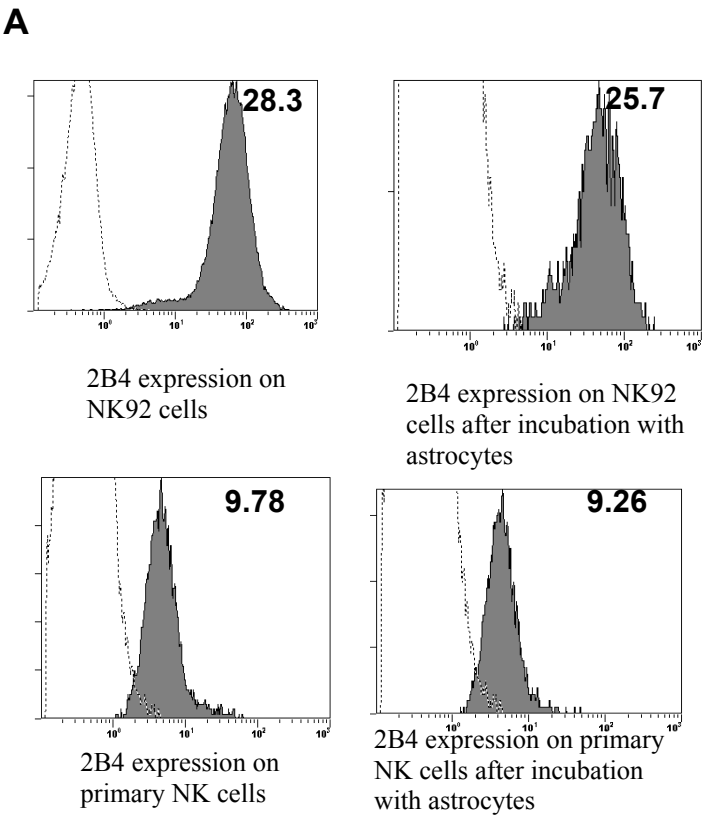

Figure 3.3 Gating strategy to separate NK cells from astrocytes. 2B4 expression did not change on NK92 cells or primary NK cells after incubation with astrocytes (A). Since astrocytes do not express 2B4, 2B4 was used to gate on NK92 and primary NK cells (B).

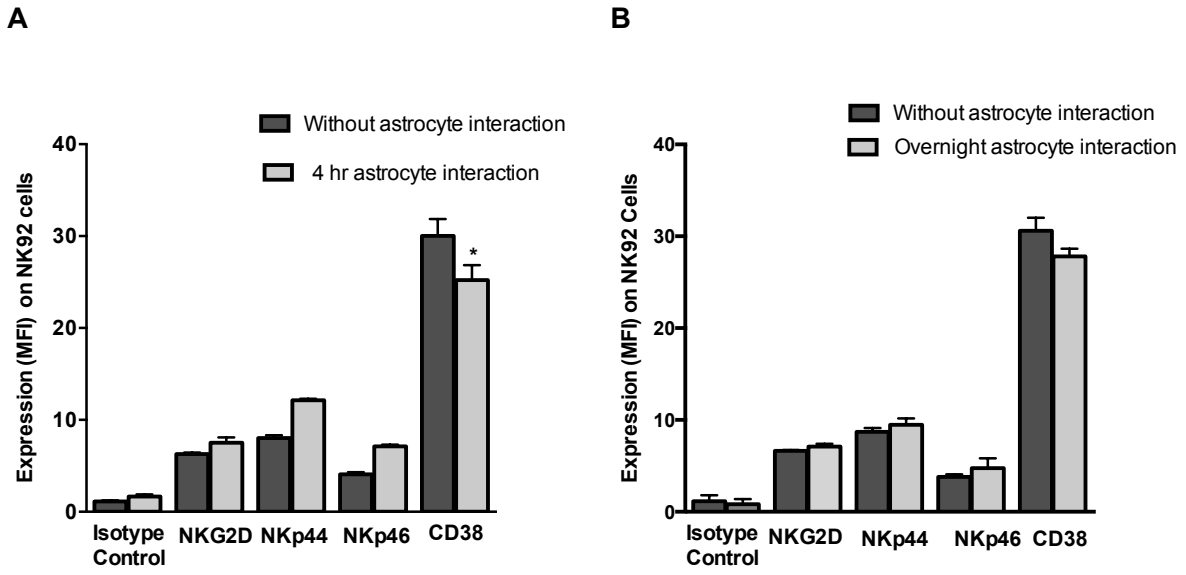

Figure 3.4 Interaction with astrocytes downregulates CD38 expression on NK92 cells. NK92 cells were co-cultured with astrocytes at a ratio of 2:1 for 4 hours (A) or overnight (B). Expression of NKG2D, NKp44, NKp46 and CD38 expression on NK92 cells cultured alone was compared to the expression on NK92 cells co-cultured with astrocytes. NK cells were separated using 2B4, as shown in figure 3.3. Mouse IgG1 was used as a negative isotype control. Figure is representative of 3 independent experiments done in duplicate. Results are expressed as mean ± SEM, * $p < 0.05$, when compared to expression on NK cells without astrocyte interaction, student's t-test

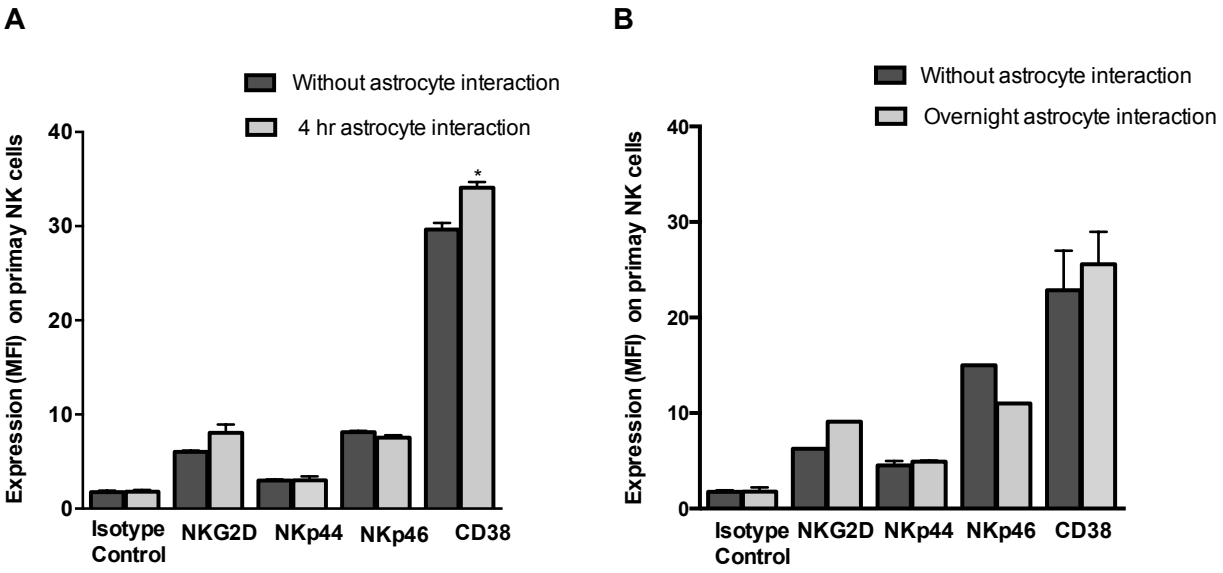

Figure 3.5 Interaction with astrocytes increases CD38 expression on primary NK cells. Human primary NK cells were co-cultured with astrocytes at a ratio of 2:1 for 4 hours (A) or overnight (B). Expression of NKG2D, NKp44, NKp46 and CD38 on primary NK cells cultured

alone was compared to expression on primary NK cells co-cultured with astrocytes. To separate NK cells from astrocytes, cells were gated on 2B4 as shown in figure 3.3. Mouse IgG1was used as a negative isotype control. Figure is representative of 3 independent experiments done in duplicate. Results are expressed as mean ± SEM, * $p < 0.05$, when compared to expression on NK cells without astrocyte interaction, student's t-test

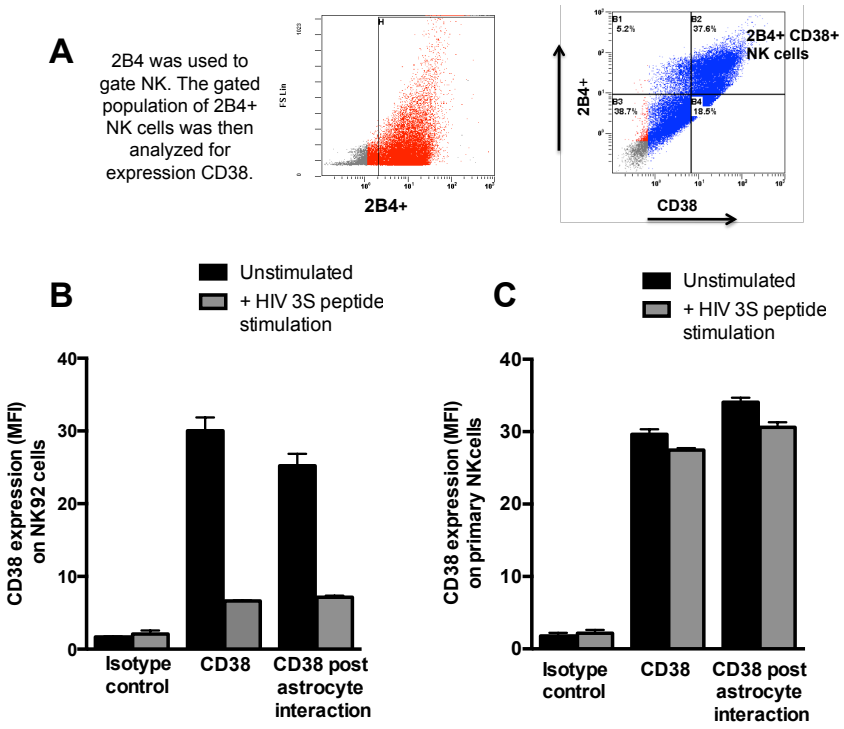

Figure 3.6 CD38 expression on NK cells does not increase with HIV 3S peptide stimulation. NK cells were co-cultured with astrocytes and 10 µg/ml HIV 3S peptide for 4 hours prior to staining with 2.5 µg anti-CD38 and anti-2B4 antibodies. To separate NK cells from astrocytes, cells were gated on 2B4 since astrocytes do not express 2B4 (A). NK CD38 expression in the presence and absence of HIV 3S peptides and astrocytes was evaluating using NK92 cells (B) and primary NK cells (C). MFIs were quantified and compared to control groups. Cells incubated with mIgG1 were used negative isotype controls. Data is representative of representative of 3 experiments done in duplicate. Results are expressed as mean ± SEM, students t test

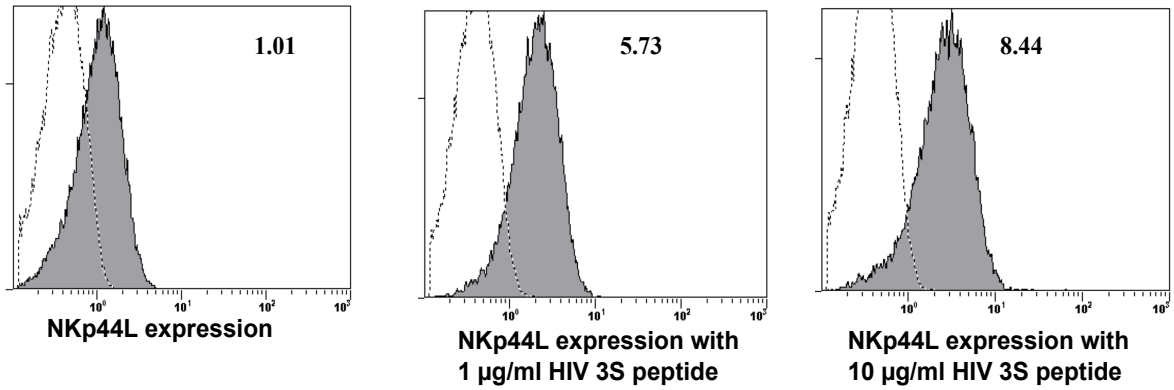

Figure 3.7 Induction of a ligand for NKp44 on Jurkat cells by HIV 3S peptide.The binding of a ligand for NKp44 (NKp44L) was established using 70 µg of NKp44-Ig fusion protein detected by anti-IgG-Fc-PE (filled histogram). Jurkat cells incubated with 2.5 µg anti-IgG-Fc-PE was used as a negative control (open histogram). Cells were tested for the binding of NKp44-Ig fustion protein with no stimulation, 1 µg/ml peptide stimulation and 10 µg/ml stimulation. MFI is indicated in the top right corner of all plots. Data is representative of 1 sample.

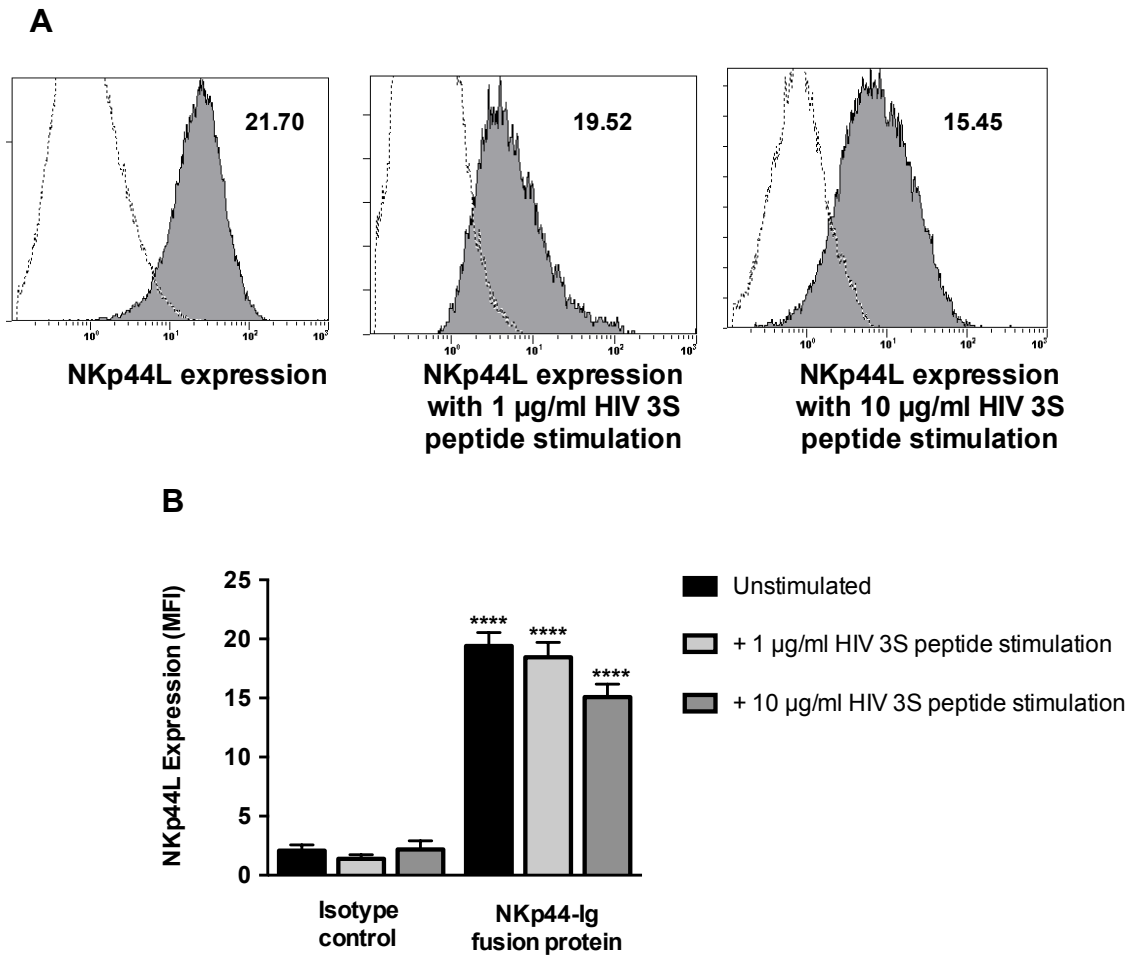

Figure 3.8 Astrocytes express a ligand for NKp44 and increasing concentrations of HIV 3S peptide decreases its expression. The binding of a ligand for NKp44 (NKp44L) on astrocytes was established using 70 µg of NKp44-Ig fusion protein detected by anti-IgG-Fc-PE (filled histogram A). Astrocytes incubated with 2.5 µg anti-IgG-Fc-PE were used as a negative controls (open histogram A). Cells were tested for the binding of NKp44-Ig fustion protein with no stimulation, 1 µg/ml peptide stimulation, and 10 µg/ml stimulation. MFI is indicated in the top right corner of all plots (A). Figure A is representative of 1 sample. Figure D the combined MFIs of tests done in duplicate on 5 different astrocyte donors. Data is represented as means ± SEM, **** $p < 0.0001$, ANOVA

CHAPTER 4

FUNCTIONAL ROLE OF CD38 AND NKP44 IN NK- ASTROCTYE INTERACTIONS IN THE PRESENCE OF HIV 3S PEPTIDE

4.1 Introduction

NK cell functions are regulated by inhibitory and activating receptors binding corresponding ligands on the surface of target cells [45]. NK cells can be recruited to the CNS following several pathological conditions [35]. NK cells could impact CNS physiology by killing astrocytes cells and by secreting IFN-γ [35]. There is little data on NK-astrocyte interactions and ligands expressed on astrocytes that could impact NK cell function. We have shown the potential of NK-astrocytes interactions through the expression of CD38 on NK cells. We have also found a novel ligand for NKp44 on astrocytes. This aim investigated if interaction through CD38 or NKp44 activates NK cell function towards astrocytes and if HIV-1 augments NK cell activity towards astrocytes.

CD38 was downregulated on NK92 cells after interaction with astrocytes, but upregulated on primary NK cells after interaction with astrocytes. Signaling through CD38 on NK cells was previously shown to induce cytotoxicity against mastocytoma cells [89] and release of IFN-γ [90]. To see if expression of CD38 inhibits or activates NK cell function, we performed ^{51}Cr release assays to test NK cell killing of astrocytes and ELISAs to quantify NK cells IFN-γ production with and without the blocking of CD38 interactions. These assays were

performed in the presence and absence of HIV 3S peptide to see if HIV 3S peptide has a protective role on astrocytes.

NKp44 is unique in expression and signal transduction. NKp44 is expressed only upon activation of NK cells and it can mediate both activating and inhibitory signals to NK cells [68, 69]. We have studied the expression and function of NCR NKp44 upon NK-astrocytes interactions in the presence or absence of an HIV 3S peptide shown to induce NK cell killing of CD4+ T cells during HIV-1 infection. Using a fusion protein consisting of the extracellular domain of NKp44 fused to Fc portion of human IgG, we determined the expression of a novel ligand for NKp44 (NKp44L) on astrocytes. Incubation of astrocytes with 3S peptide downregulated NKp44L expression on astrocytes. To see if this downregulation of NKp44L on astrocytes protects astrocytes from NK cell functions we performed a ^{51}Cr release assay to test the killing of astrocytes with NKp44 interactions blocked in the presence and absence of HIV 3S peptide. The release of IFN-γ from NK cells co-cultured with astrocytes in the presence of NKp44 blocking antibodies and HIV 3S peptide was quantified using an ELISA.

4.2 Results

A. Functional Role of CD38 in NK-Astrocyte Interactions

Blocking CD38 interactions inhibits NK92 cell killing of astrocytes

CD38 is expressed on both astrocytes and NK cells [85, 86]. To determine if signaling through CD38 on NK92 cells or astrocytes activates the killing of astrocytes by NK92 cells, the lysis of astrocytes cells by NK92 cells was quantified by a standard ^{51}Cr release assay. Since astrocytes and NK92 cells both express CD38, blocking of CD38 using anti-CD38 antibody was done separately on each cell type and then on both cell types together. Astrocytes incubated with

NK92 cells at varying target to effector cell ratio for 4 hours. Level of killing was compared to astrocytes cells incubated with 0.5 mg/ml mIgG1 isotype antibody or no antibody, which served as a positive control (no blocking) of cell lysis under unblocked conditions (Figure 4.1 A). Alternatively, NK92 cells were incubated with 0.5 mg/ml anti-CD38 or mIgG1 isotype control antibody prior to incubation with astrocytes incubated with no antibody (Figure 4.1 B) or 0.5 mg/ml anti-CD38 antibody (Figure 4.1C). Blocking of CD38 on astrocytes did not have any effect on NK cell mediated lysis of astrocytes (Figure 4.1 A). However, the blocking of CD38 on NK92 cells (Figure 4.1 B) significantly decreased NK cell killing of astrocytes at effector:target cell ratios 20:1 as compared to the control (Figure 4.1 B). The blocking of CD38 interactions on both NK92 and astrocytes (Figure 4.1 C) significantly decreased NK92 cell killing of astrocytes at effector:target cell ratios 20:1 as well when compared to the control. However, when compared to the lysis of astrocytes without anti-CD38, there was no significant difference in killing. Thus, the blocking of CD38 on astrocytes seems to have no effect on NK92 cell function, whereas the blocking of CD38 on NK92 cells decreases NK cell killing of astrocytes.

Blocking of CD38 interactions in the presence of HIV 3S peptide further decreases NK92 cell lysis of astrocytes

To determine if HIV 3S peptide augmented or decreased this inhibition of NK cell cytotoxicity towards astrocytes with CD38 interactions blocked, astrocytes were stimulated overnight with 10 µg/ml HIV 3S peptide before being loaded with chromium (Figure 4.2). Blocking of CD38 on NK92 cells significantly decreased the killing of HIV 3S peptide stimulated astrocytes at an effector:target ratio of 20:1 (Figure 4.2 A). The overall killing of astrocytes slightly decreased with HIV 3S peptide stimulation as compared to the killing of

astrocytes without peptide stimulation (Figure 4.2 B), but the decrease was not statistically significant.

Blocking of CD38 interactions inhibits primary NK cell killing of astrocytes

In order to determine whether blocking CD38 interactions on primary human NK cells inhibits human primary NK cell cytotoxic function, we isolated primary NK cells from PBMCs of healthy individuals. The primary NK cells were cultured in recombinant human IL-2. The experiments were conducted under the same specifications as for NK92 cells except the target to effector cell ratios were 10:1, 5:1, and 2.5:1. The blocking of CD38 on primary NK cells inhibited NK cell cytotoxic function when compared cells that received the isotype control or no blocking treatment (Figure 4.3). This decrease in astrocyte lysis was statistically significant at effector:target cell ratios of 10:1 and 5:1.

Blocking of CD38 interactions decreases primary NK cell lysis of HIV 3S peptide stimulated astrocytes

To determine if HIV 3S peptide decreased this inhibition on primary NK cells, astrocytes were stimulated overnight with 10 μg/ml HIV 3S peptide before being loaded with chromium and incubated with primary NK cells (Figure 4.4). Blocking of CD38 on primary NK cells also significantly inhibited NK cell lysis of astrocytes at effector:target ratios of 10:1 and 5:1 (Figure 4.4 A). HIV 3S peptide overall decreased NK cell lysis of astrocytes, but the decrease was not statistically significant (Figure 4.4 B).

Blocking CD38 decreased IFN-γ production of NK- astrocyte co-cultures

IFN-γ levels were determined from astrocyte-NK cell co-culture supernatants by an IFN-γ ELISA. NK92 cells and astrocytes were first Fc blocked. NK92 cells were then treated with 2.5 μg anti-CD38 and then incubated with astrocytes in the absence (Figure 4.5 A) and

presence of HIV 3S peptide (Figure 4.5 B). NK92 cells with no antibody treatment or with 2.5 µg mIgG1 were used as controls. Astrocytes and NK92 cells treated with anti-CD38, mIgG1 isotype control or no antibody were cultured alone and compared to the co-culture levels. Both NK cells and astrocytes express CD38 and can release IFN-γ. IFN-γ release of astrocytes and NK cells cultured alone with anti-CD38, mIgG1 isotype control or no antibody treatment was quantified and these values were subtracted from the IFN-γ release of astrocytes and NK cells co-cultured together. Overall, NK92 cells incubating with astrocytes increased interferon gamma production. Blocking CD38 on co-cultures of NK92 cells and astrocytes significantly decreased IFN-γ (Figure 4.5 A). Stimulation with HIV 3S peptide had no effect on IFN-γ production even with CD38 blocked (Figure 4.B) as compared to IFN-γ without HIV 3S peptide stimulation.

B. Functional Role of NKp44 in NK – Astrocyte Interactions

Blocking of NKp44 interactions protects astrocytes from NK92 cell killing

We found a ligand for NKp44 expressed on primary human fetal astrocytes. NKp44 interactions can be activating or inhibitory depending on the ligand it binds [68, 69]. To establish if the ligand for NKp44 on astrocytes is inhibitory or activating, NK killing of astrocytes was tested with the blocking of NKp44 interactions and compared to the killing of astrocytes that did not receive any blocking antibodies. NKp44 interactions were blocked by either blocking NKp44 on NK cells using anti-NKp44 or by blocking NKp44L on astrocytes using NKp44-Ig fusion protein. Astrocytes were loaded with ^{51}Cr and incubated with 1.0 µg/µl NKp44-Ig fusion protein (Figure 4.6 A). Astrocytes were then incubated with NK92 cells at varying target to effector cell ratio. Level of killing was compared to astrocytes cells incubated without antibody, which served as a positive control (no blocking) of cell lysis under unblocked conditions. Alternatively, NK92

cells were incubated with 0.5 mg/ml anti-NKp44 or mIgG1 isotype control antibody prior to incubation with astrocytes incubated with no antibody (Figure 4.6 B). Blocking of NKp44L on astrocytes decreased NK cell killing significantly at the 20:1 effector:target cell ratio when compared to cells without any blocking (Figure 4.6 A). The blocking on NKp44 on NK cells also significantly decreased NK cell killing at the 20:1 effector:target cell ratio compared to cells that were treated with mIgG1 isotype control or cells that received no blocking treatment (Figure 4.6 B).

HIV 3S peptide stimulation protects astrocytes from NK92 cell killing and the blocking of NKp44 further protects HIV 3S stimulated astrocytes from NK92 cell killing

To determine if blocking NKp44 interactions further protected HIV 3S peptide astrocytes from NK cell attack, astrocytes were stimulated overnight with 10 μg/ml HIV 3S peptide before being labeled with chromium (Figure 4.7). NKp44 interactions were blocked by blocking NKp44L on astrocytes (Figure 4.7 A) or by blocking NKp44 receptor on NK cells (Figure 4.7 B). Blocking NKp44 signaling significantly inhibited the lysis of HIV 3S peptide stimulated astrocytes (Figure 4.7 A, B). Stimulation with HIV 3S peptide further protected astrocytes from NK cell killing as compared to unstimulated astrocytes, but the decrease in killing the presence of HIV 3S peptide was not statistically significant (Figure 4.7 C).

Blocking of NKp44 interactions on primary NK cells inhibits NK cell killing of astrocytes

In order to determine whether blocking NKp44 interactions on primary human NK cells inhibits human primary NK Cell cytotoxic function, we isolated primary NK cells from PBMCs of healthy individuals. The primary NK cells were cultured in recombinant human IL-2. The experiments were conducted under the same specifications as for NK92 cells except the target to effector cell ratios were 10:1, 5:1, and 2.5:1. The blocking of NKp44L on astrocytes significantly

decreased their lysis by on primary NK cells (Figure 4.8 A) at the 10:1 and 5:1 effector:target cell ratio. The blocking of NKp44 on primary NK cells also significantly inhibited NK cell cytotoxic function when compared cells that received the isotype control or no blocking treatment (Figure 4.8 B).

Blocking of NKp44 interactions on primary NK cells decreases NK cell lysis of HIV 3S peptide stimulated astrocytes.

To see if HIV 3S peptide decreased this inhibition on primary NK cells, astrocytes were stimulated overnight with 10 μg/ml HIV 3S peptide before being loaded with chromium and incubated with primary NK cells (Figure 4.9). Blocking of NKp44L on astrocytes (Figure 4.9 A) or the blocking of NKp44 on primary NK cells (Figure 4.9 B) also significantly inhibited NK cell lysis of astrocytes. HIV 3S peptide overall decreased NK cell lysis of astrocytes, but the decrease was not statistically significant (Figure 4.9 C).

Blocking NKp44 decreased IFN-γ production of NK- astrocyte co-cultures and the presence of HIV 3S peptide did not increase or decrease IFN-γ production of NKp44 blocked cells

IFN-γ levels were determined from astrocyte-NK cell co-culture supernatants by an IFN-γ ELISA. NK92 cells and astrocytes were first Fc blocked. NK92 cells were then treated with 2.5 μg anti-NKp44 and then incubated with astrocytes at a ratio of 10:1 in the absence (Figure 4.10 A) and presence of HIV 3S peptide (Figure 4.10 B). NK92 cells with no antibody treatment or with 2.5 μg mIgG1 were used as controls. Astrocytes and NK92 cells treated with mIgG1 isotype control or received no antibody treatment were cultured alone and compared to the co-culture levels. Both NK cells can release IFN-γ. IFN-γ release of astrocytes and NK cells cultured alone with anti-CD38, mIgG1 isotype control or no antibody treatment was quantified

and these values were subtracted from the IFN-γ release of astrocytes and NK cells co-cultured together. Overall, NK92 cells incubating with astrocytes increased IFN-γ production. Blocking NKp44 on NK92-astrocyte co-cultures decreased IFN-γ production (Figure 4.10 A). Stimulation with HIV 3S peptide had no effect on IFN-γ even with NKp44 blocked (Figure 4.10 B).

4.3 Conclusions

Our data demonstrated that NK cells have the ability to kill astrocytes. Previous studies reported signaling through CD38 on NK cells induces cytotoxicity against mastocytoma cells [89] and the release the release of IFN-γ [90]. Blocking CD38 interactions in our studies interactions significantly decreased NK cell mediated lysis of astrocytes and decreased the IFN-γ release. From this study we cannot conclude what ligand on astrocytes CD38 is acting on. Future studies using anti-CD31 antibody or CD31 fusion protein will confirm the CD38 ligand on astrocytes.

Using a NKp44 fusion protein, we found the expression of a novel ligand for NKp44 on astrocytes. NKp44 interactions can be activating or inhibitory depending on the ligand it binds [68, 69]. In our studies, blocking NKp44 interactions by using a NKp44-Ig fusion protein to block NKp44L on astrocytes or by using anti-NKp44 to block NKp44 receptor on NK cells decreased lysis of astrocytes by NK92 cells and primary NK cells, suggesting that the ligand for NKp44 on astrocytes is activating. Incubation of astrocytes with 3S peptide downregulated NKp44L expression on astrocytes implicating protection from NK mediated killing. Thus, our study showed that blocking NKp44 and CD38 signaling decreased NK cell killing of astrocytes and that HIV 3 S peptide has a protective effect on astrocytes from NK cell mediated killing during HIV infection and impacts astrocyte role in HAND.

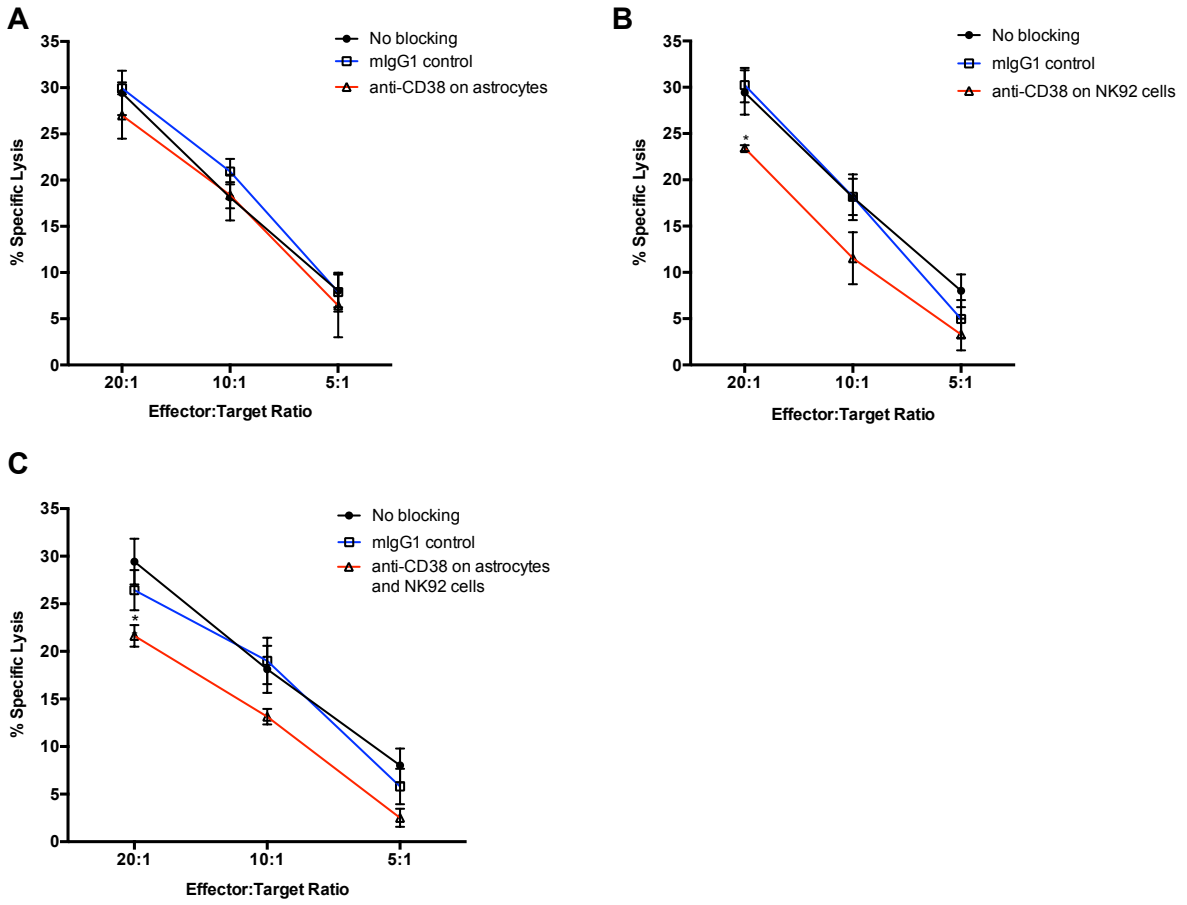

Figure 4.1 Blocking CD38 interactions inhibits NK92 cell cytotoxicity. Astrocytes and NK92 cells were first blocked with Human IgG Fc fragment to prevent unspecific binding and antibody dependent cellular cytotoxicity. Astrocytes were loaded with ^{51}Cr and incubated with either 0.5 mg/ml anti-CD38, 0.5 mg/ml mIgG1 isotype antibody or no antibody. Astrocytes were then incubated with NK92 cells at varying target to effector cell ratios for 4 hours at 37°C. Level of killing was compared to astrocytes incubated with 0.5 mg/ml mIgG1 isotype antibody or no antibody, which served as a positive control (no Blocking) of cell lysis under unblocked conditions (A). Alternatively, NK92 cells were incubated with 0.5 mg/ml anti-CD38 or mIgG1 isotype control antibody prior to incubation with astrocytes incubated with either no antibody (B) or 0.5 mg/ml anti-CD38 antibody (C). Figure is representative of 3 independent experiments performed in triplicate. Data is displayed as means ± SEM. * $p < 0$ .05, ANOVA.

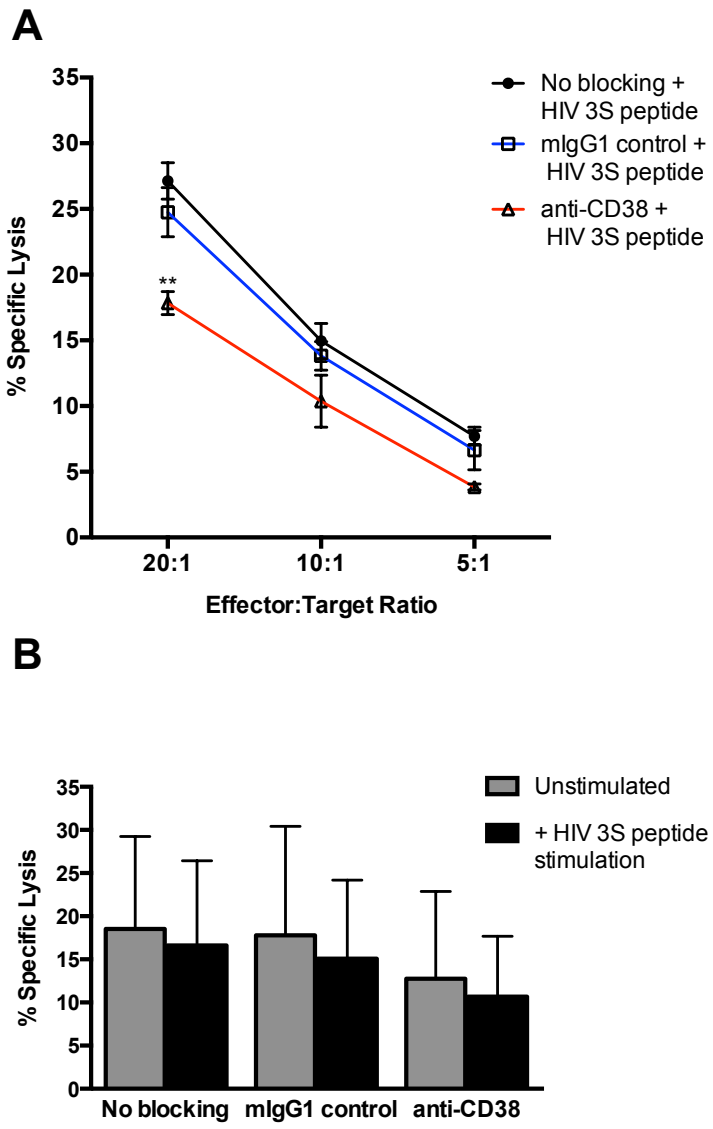
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Figure 4.2 Blocking CD38 interactions on NK92 cells decreases NK cell lysis of HIV 3S peptide stimulated astrocytes. Astrocytes that received 10 µg/ml 3S peptide and NK92 cells were first blocked with Human IgG Fc fragment to prevent unspecific binding and antibody dependent cellular cytotoxicity. Astrocytes were loaded with ^{51}Cr. Astrocytes were then incubated with NK92 cells that received either 0.5 mg/ml anti-CD38, 0.5 mg/ml mIgG1 isotype control or no blocking treatment at varying target to effector cell ratios for 4 hours at 37°C (A). NK92 cell lysis of HIV 3S peptide stimulated astrocytes was compared to NK92 cell lysis in the absence of HIV 3S peptide (B). Figure is representative of 3 independent experiments performed in triplicate. Data is displayed as means ± SEM. ** $p < 0.01$, ANOVA.

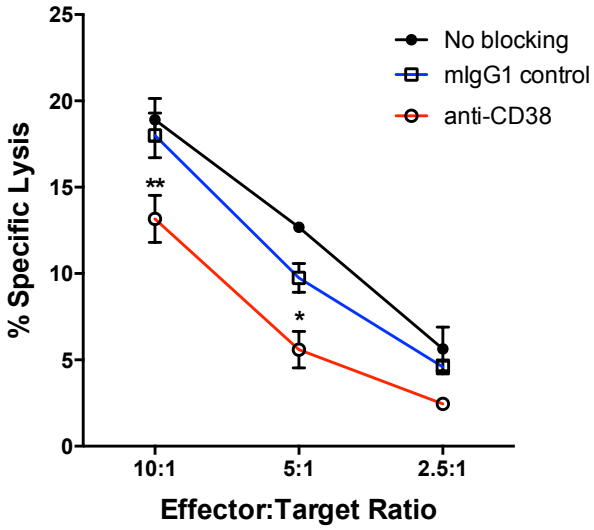

Figure 4.3 Blocking CD38 interactions inhibits primary NK cell cytotoxicity. Astrocytes and primary NK cells were first blocked with Human IgG Fc fragment to prevent unspecific binding and antibody dependent cellular cytotoxicity. Astrocytes loaded with ^{51}Cr were then incubated with primary NK cells at varying target to effector cell ratios for 4 hours at 37°C. NK92 cells were incubated with 0.5 mg/ml anti-CD38. Level of killing was compared to primary NK cells incubated with 0.5 mg/ml mIgG1 isotype antibody or no antibody, which served as a positive control (no blocking) of cell lysis under unblocked conditions. Figure is representative of three independent experiments performed in triplicate. Data is displayed as means ± SEM.* $p < 0.05$, ** $p < 0.01$, ANOVA.

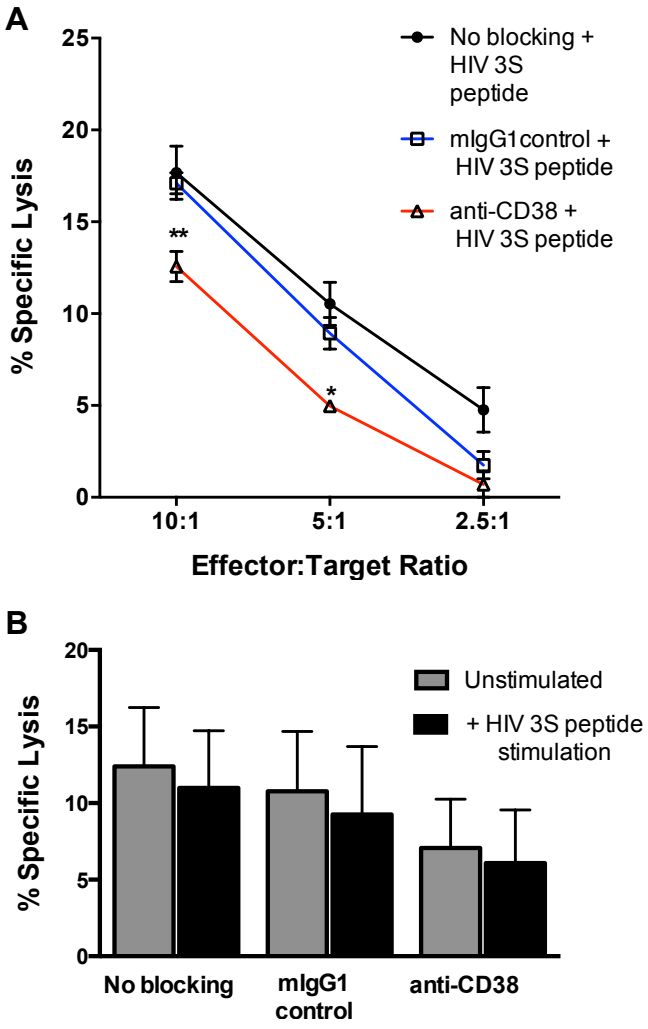

Figure 4.4 Blocking CD38 interactions on primary NK cells decreases NK cell lysis of HIV 3S peptide stimulated astrocytes. Astrocytes treated with 10 µg/ml HIV 3S peptide and primary NK cells were first blocked with Human IgG Fc fragment to prevent unspecific binding and antibody dependent cellular cytotoxicity. Astrocytes loaded with ^{51}Cr were then incubated with primary NK cells at varying target to effector cell ratios for 4 hours at 37°C. Primary NK cells were incubated with 0.5 mg/ml anti-CD38 or mIgG1 antibody prior to incubation with astrocytes. Level of killing was compared to primary NK cells incubated with 0.5 mg/ml mIgG1 isotype antibody or no antibody, which served as a positive control (no blocking) of cell lysis under unblocked conditions (A). Primary NK cell lysis of HIV 3S peptide stimulated astrocytes compared to primary NK cell lysis in the absence of HIV 3S peptide (B). Figure is representative of 3 independent experiments performed in triplicate. Data is displayed as means ± SEM. * $p < 0.05$, ** $p < 0.01$, ANOVA.

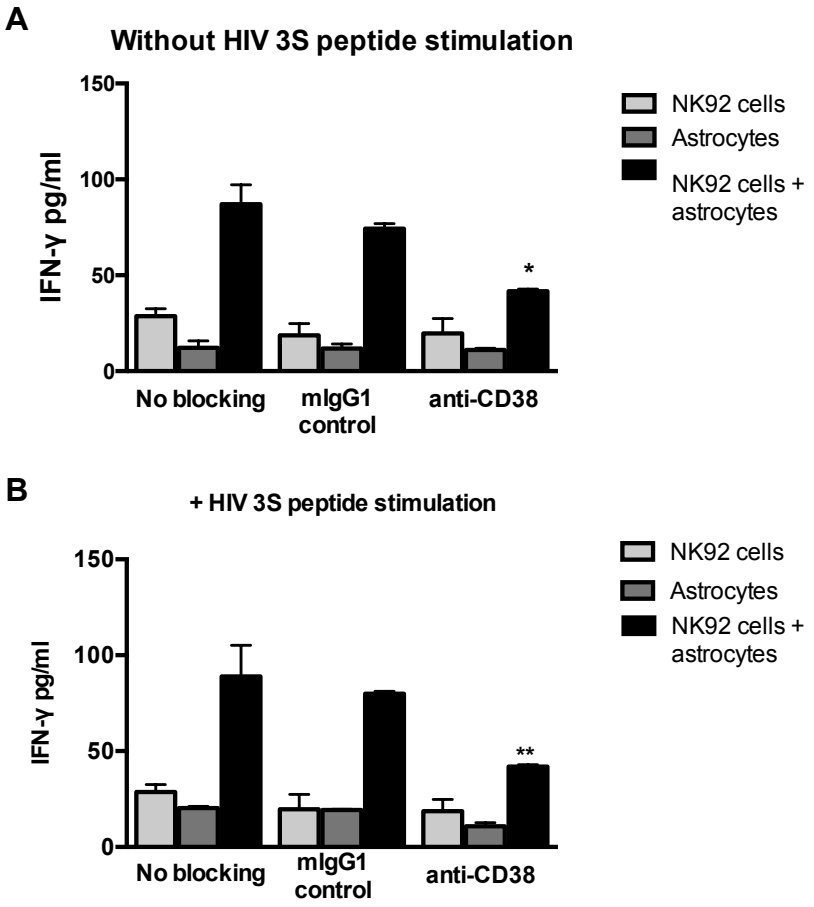

Figure 4.5 Blocking CD38 signaling decreases the release of IFN-γ from NK cell – astrocyte co-cultures. IFN-γ levels were determined from astrocyte-NK cell co-culture supernatants by an IFN-γ ELISA. NK92 cells and astrocytes were first Fc blocked. NK92 cells were then treated with 2.5 µg anti-CD38 and then incubated with astrocytes in the absence (A) and presence of HIV 3S peptide (B). NK92 cells with no antibody treatment or with 2.5 µg mIgG1 were used as controls. Astrocytes and NK92 cells treated with 1 µg anti-CD38, mIgG1 isotype control or received no antibody treatment were cultured alone and compared to the co-culture levels. Figure is representative of 2 independent experiments performed in triplicate. Data is displayed as means ± SEM. * $p < 0.05$,** $p < 0.01$, ANOVA.

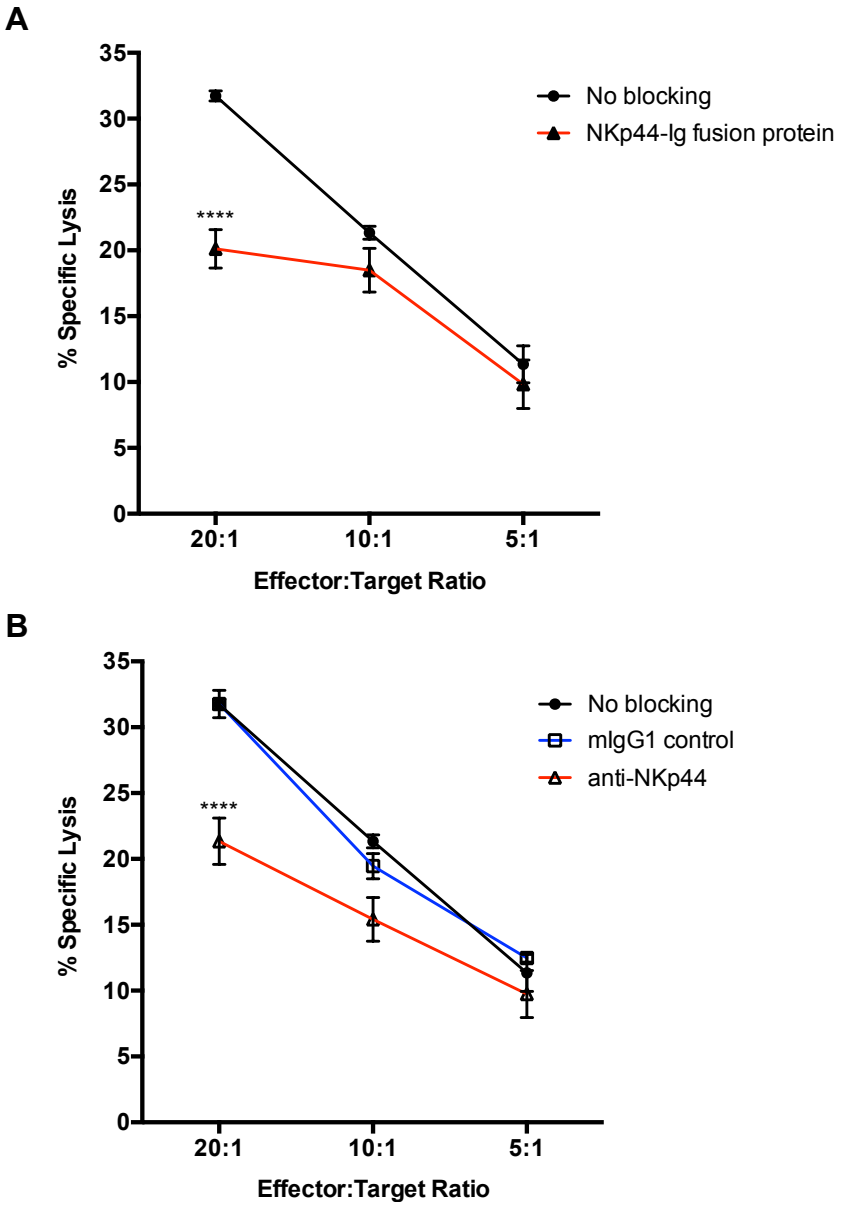

Figure 4.6 Blocking NKp44 interactions inhibits NK92 cell cytotoxicity of astrocytes. Astrocytes and NK92 cells were first blocked with Human IgG Fc fragment to prevent unspecific binding and antibody dependent cellular cytotoxicity. Astrocytes were loaded with ^{51}Cr and incubated with 1.0 µg/µl NKp44-Ig fusion (A). Astrocytes were then incubated with NK92 cells at varying target to effector cell ratios for 4 hours at 37°C. Level of killing was compared to astrocytes cells incubated with no antibody, which served as a positive control (no blocking) of cell lysis under unblocked conditions. Alternatively, NK92 cells were incubated with 0.5 mg/ml anti-NKp44 or mIgG1 isotype control antibody prior to incubation with astrocytes incubated with no antibody (B). Figure is representative of 3 independent experiments performed in triplicate. Data is displayed as means ± SEM. **** $p < 0.0001$, ANOVA.

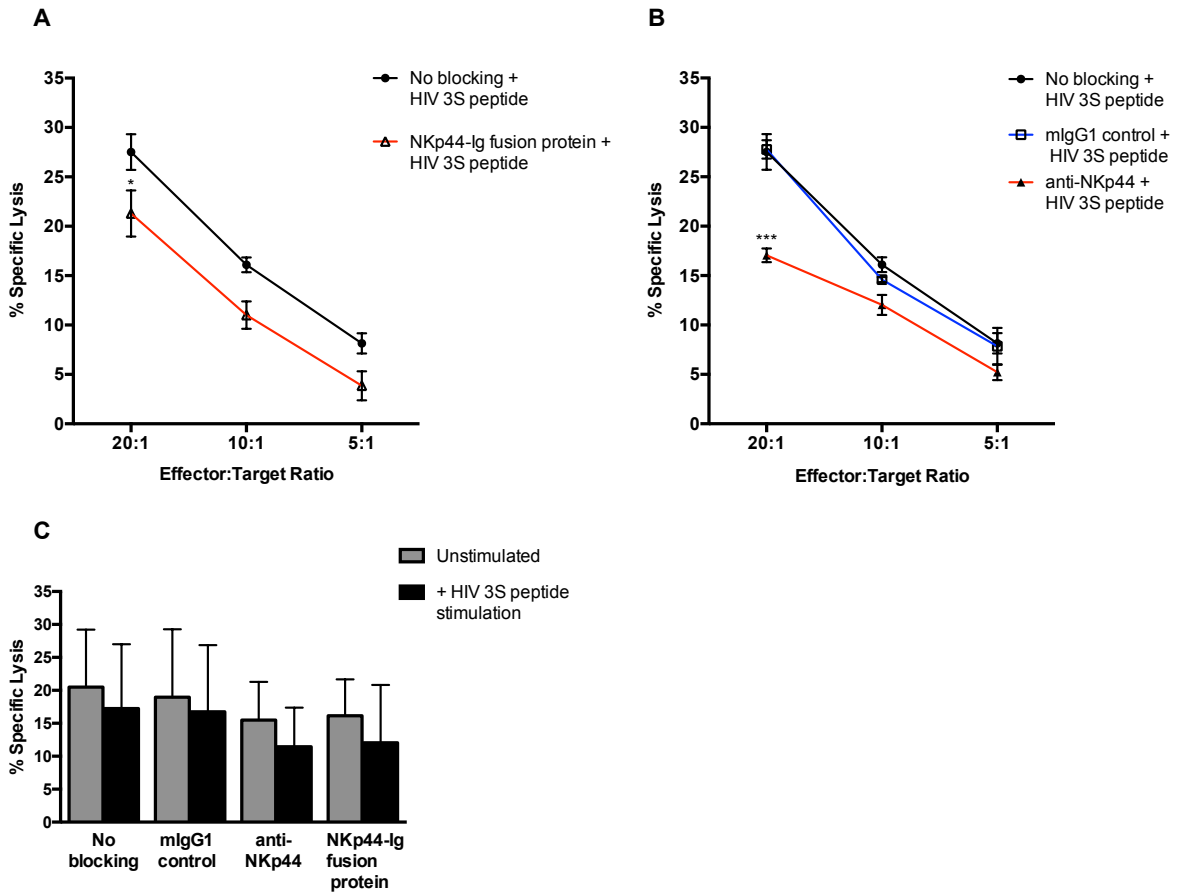

Figure 4.7 Blocking NKp44 interactions further protects HIV 3S peptide stimulated astrocytes from NK92 cell killing. Astrocytes and NK92 cells were first blocked with Human IgG Fc fragment to prevent unspecific binding and antibody dependent cellular cytotoxicity. Astrocytes stimulated with 10 µg/ml HIV 3S peptide were loaded with ^{51}Cr and incubated with 1.0 µg/µl NKp44-Ig fusion protein (A). NK92 cells that received 0.5 NKp44 or mIgG1 isotype control antibody were incubated at varying ratios with HIV 3S peptide stimulated astrocytes loaded with ^{51}Cr for 4 hours (B). Overnight stimulation with 10 µg/ml HIV 3S peptide on astrocytes decreased NK cell killing of astrocytes when compared to astrocytes with no peptide stimulation (C). Figure is representative of 3 independent experiments performed in triplicate. Data is displayed as means ± SEM. * $p < 0.05$, *** $p < 0.001$, ANOVA.

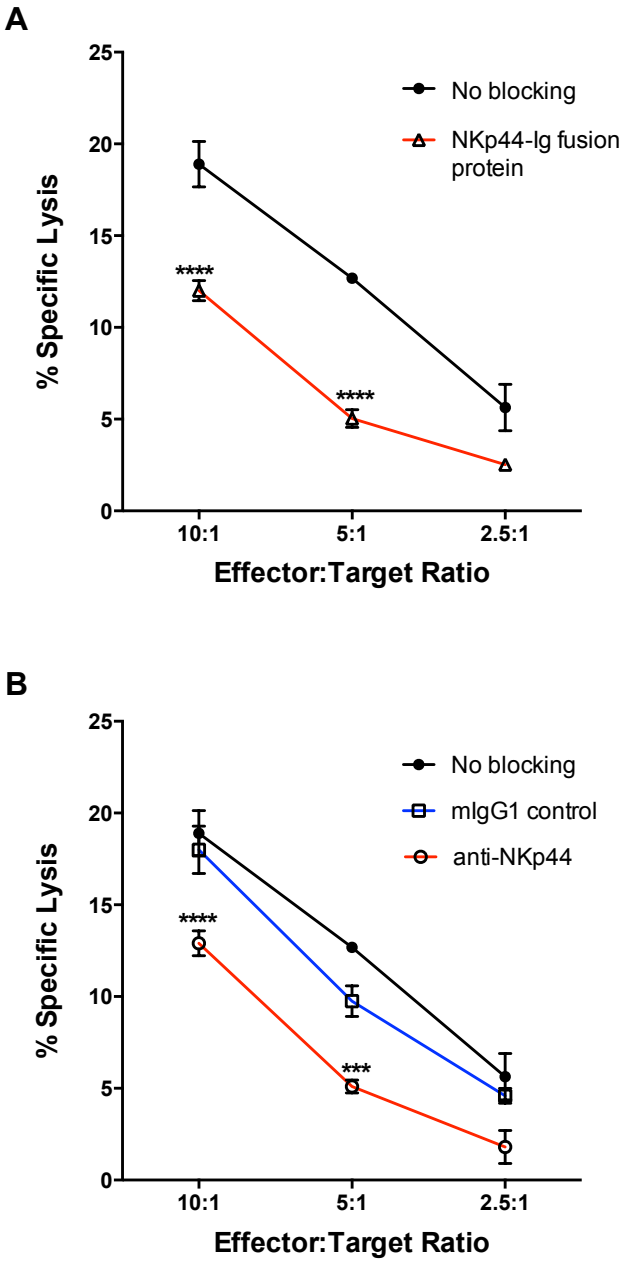

Figure 4.8 Blocking NKp44 interactions inhibits primary NK cell cytotoxicity of astrocytes. Astrocytes and primary human NK cells were first blocked with Human IgG Fc fragment to prevent unspecific binding and antibody dependent cellular cytotoxicity. Astrocytes were loaded with ^{51}Cr and incubated with 1.0 µg/µl NKp44-Ig fusion (A). Astrocytes were then incubated with primary NK cells at varying target to effector cell ratios for 4 hours at 37°C. Level of killing was compared to astrocytes cells incubated with no antibody, which served as a positive control (no blocking) of cell lysis under unblocked conditions. Alternatively, NK92 cells were incubated with 0.5 mg/ml anti-NKp44 or mIgG1 isotype control antibody prior to incubation with astrocytes incubated with no antibody (B). Figure is representative of 3 independent experiments performed in triplicate. Bars ± SEM. *** $p < 0.001$, **** $p < 0.0001$, ANOVA.

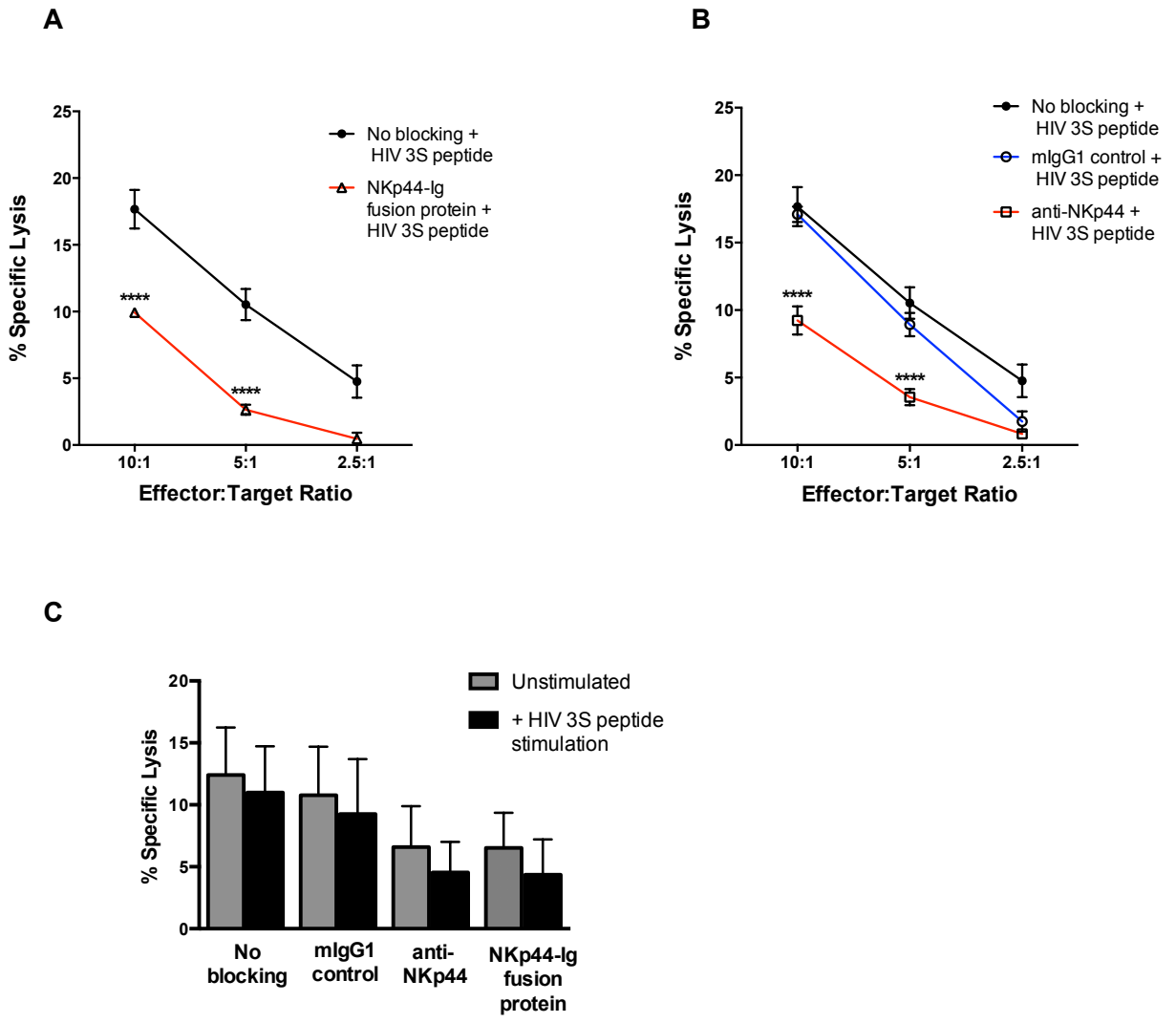

Figure 4.9 Blocking NKp44 interactions further protects HIV 3S peptide stimulated astrocytes from primary NK cell killing. Astrocytes and primary NK cells were first blocked with Human IgG Fc fragment to prevent unspecific binding and antibody dependent cellular cytotoxicity. Astrocytes stimulated with 10 µg/ml HIV 3S peptide were loaded with ^{51}Cr and then incubated with 1.0 µg/µl NKp44-Ig fusion (A). Astrocytes that received no blocking treatment were used as a control. Alternatively, primary NK cells that received 0.5 mg/ml NKp44 or mIgG1 isotype control antibody were incubated at varying ratios with HIV 3S peptide stimulated astrocytes loaded with ^{51}Cr (B). Overnight stimulation with 10 µg/ml HIV 3S peptide on astrocytes had no effect on NK cell killing of astrocytes when compared to astrocytes with no peptide stimulation (C). Figure is representative of 3 independent experiments performed in triplicate. Bars ± SEM. **** $p < 0.0001$, ANOVA.

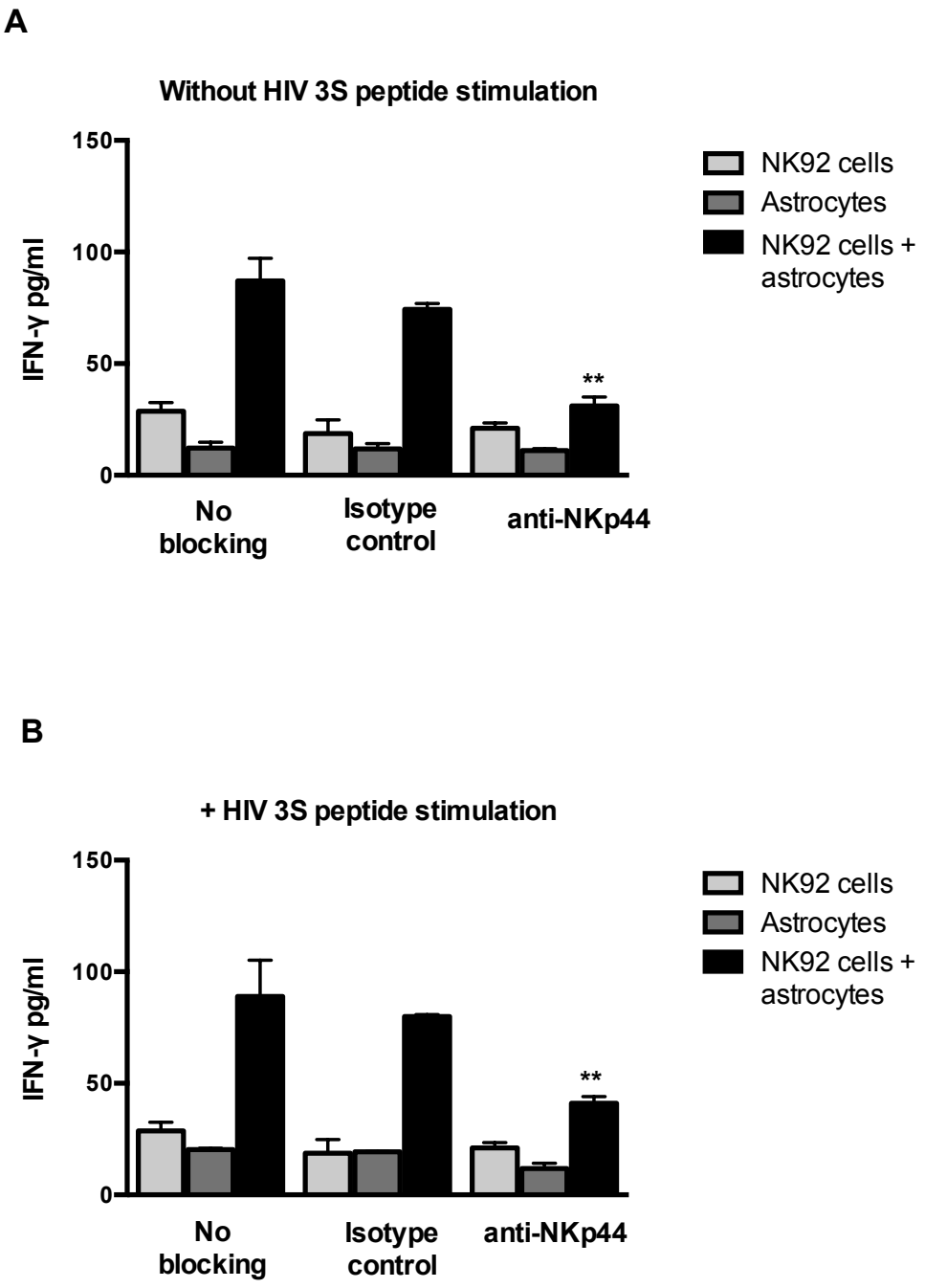

Figure 4.10 Blocking NKp44 signaling decreases the release of IFN-γ. IFN-γ levels were determined from astrocyte-NK cell co-culture supernatants by an IFN-γ ELISA. NK92 cells and astrocytes were first Fc blocked. NK92 cells were then treated with 2.5 μg anti-CD38 and then incubated with astrocytes in the absence (A) and presence of HIV 3S peptide (B). NK92 cells with no antibody treatment or with 2.5 μg mIgG1 were used as controls. Astrocytes with and without HIV 3S peptide stimulation were treated with 1 μg anti-CD38, mIgG1 isotype control or received no antibody treatment. Figure is representative of 2 independent experiments performed in triplicate. Bars ± SEM , ** $p < 0.01$, ANOVA.

CHAPTER 5

DISCUSSION & SUMMARY

5.1 Discussion

NK cells can be recruited to the CNS following several pathological conditions such as HAND [35]. Because both HIV-1 binding and infection can affect astrocyte function, astrocytes have a strong pathogenic potential for being intimately involved in HAND. The interactions between NK cells and CNS glial cells, especially astrocytes, are understudied. NK cells could impact CNS physiology by killing glial cells and by secreting IFN-γ. NK cell functions are regulated by inhibitory and activating receptors binding corresponding ligands on the surface of target cells [42, 45, 47]. Previous studies reported the ability of NK cells to kill astrocytes [54, 73, 103]. Their mechanism of killing action was not previously investigated and no prior studies have investigated NK-astrocyte interactions in the context of HAND. This study aimed to identify NK-associated ligands expressed by human astrocytes that confer this NK-directed cytotoxicity of astrocytes and assay the cytotoxicity differences in presence and absence of HIV 3S peptide.

We first surveyed astrocytes for NK associated molecules important in NK cell signaling. NCRs play critical role in the cytolytic function of NK cells. Among the NCRs, NKp44 is unique in expression and signal transduction. NKp44 is expressed only upon activation of NK cells and it can mediate both activating and inhibitory signals to NK cells [54]. The ligand for NKp44L has only been previously reported on infected or transformed cells [70, 71]. Using a fusion

protein consisting of the extracellular domain of NKp44 fused to Fc portion of human IgG, we determined healthy human fetal astrocytes express a novel ligand for NKp44. This study is the first report of NKp44L expression on healthy cells. To test if this novel ligand for NKp44 is activating or inhibitory, NK cell killing of astrocytes and IFN-γ production was tested with and without the blocking of NKp44 interactions. Blocking NKp44 interactions decreased NK cell killing of astrocytes and IFN-γ production, implicating NKp44 interaction with NKp44L on astrocytes activates NK cell function.

Astrocytes can also secrete cytokines that may affect expression of receptors on NK cells [18]. In this study we evaluated receptor expression on NK cells after interaction with astrocytes. CD38 is expressed on both NK cells and astrocytes and has an important implication in HIV-1 infection [31, 32]. CD38 was increased on primary NK cells after incubation with astrocytes. In contrast, CD38 expression was decreased on NK92 cells after incubation with astrocytes. The NK cells and astrocytes incubated together for 4 hours or overnight. Overnight incubations resulted in too many dead cells. The induction of receptors and increase in expression of receptors may take longer than 4 hours. From this data we cannot conclude if the astrocytes are secreting cytokines that alter expression on NK cells or if the alteration of CD38 expression is from cell-cell contact. To assess dependency on soluble factors as opposed to direct cell-cell contact future studies can repeat this experiment again quantifying CD38 expression on NK cells in Transwells vs. non-Transwell co-cultures with astrocytes.

Signaling through CD38 on NK cells induces cytotoxicity against mastocytoma cells [89] and the release of IFN-γ [90]. CD38 mediated NK cytotoxicity towards astrocytes has previously never been evaluated. We found that regardless of the increase or decrease in CD38 expression, blocking of CD38 decreases NK92 cell and primary NK cells killing of astrocytes. Blocking

CD38 also decreased IFN-γ production. CD31 is the natural ligand for CD38, however, recent studies discovered a novel ligand for CD38 on dendritic cells that is not CD31 [87]. CD31 is normally found on endothelial cells, platelets, macrophages, granulocytes, NK cells, T cells, and, neutrophils [87]. Future studies will need to investigate the expression of CD31 on astrocytes after co-culture with NK cells. Using a CD38 fusion protein, the ligand for CD38 on astrocytes could be identified.

Both astrocytes and NK cells play roles in HIV-1 infection. No previous studies have reported NK cell function of HIV-1 infected astrocytes or NK cells role in HAND. CD38 and NKp44L expression have implications in HIV-1 infection. Previous studies reported an increase in CD38 expression in activated astrocytes stimulated with HIV gp120 [31]. HIV gp120 and HIV gp41 are envelope proteins crucial to HIV entry into target cells [2]. The effect of HIV gp41 on astrocytes has not previously been studied. We studied the context of HIV-1 infection in the CNS using a highly conserved HIV gp41 3S peptide that is essential for HIV-1 entry into target cells. We evaluated CD38 expression on NK cells, NK killing of astrocytes, and NK cell production of IFN-γ in the presence and absence of HIV 3S peptide stimulated astrocytes. We found no difference in the expression of CD38. Previous studies determined HIV 3S peptide induced the expression of NKp44L on T cells and expression of NKp44L increased with increasing concentration of HIV 3S peptide [6]. We stimulated astrocytes with increasing concentration of 3S peptide, and strikingly, found that NKp44L expression on astrocytes decreased with increasing HIV 3S peptide concentrations. This decrease in NKp44L expression decreased NK mediated cytotoxicity towards astrocytes, suggesting that NKp44 has a protective effect on astrocytes during HIV-1 infection. More studies will need to be conducted in order to determine the function of HIV 3S peptide on astrocytes. Since the decrease in NK killing of astrocytes with

HIV 3S peptide stimulation was not statistically significant, future studies could involve using higher than 10 μg/ml concentration and using other HIV-1 peptides or astrocytes infected with a pseudotyped HIV-1 in order to determine NK killing of astrocytes infected with HIV. These modifications suggest NK cells may play a possible role in the pathogenesis of HAND by killing astrocytes. NK cells could either promote or limit HAND by their intrinsic immune responses. Many unanswered questions still remain, and the future challenge is to delineate the precise influence of NK cells on CNS pathology in context of HAND.

5.2 Limitations

As with any research conducted using human cells, there were potential limitations encountered in this study. Most data was generated using astrocytes from 3 different astrocyte donors. A larger sample size would more reliably reflect the population mean and could increase significance. The astrocytes and NK cells came from different donors, so killing of healthy astrocytes could possibly be due to recognition of non-self. Another major limitation of this study is that healthy adult astrocytes could differ in NKp44L expression than healthy fetal astrocytes, but due to obvious ethical and practical considerations, there is no availability of healthy adult primary astrocytes.

5.3 Conclusion

In conclusion, our studies reported NK cell killing of healthy astrocytes. Although, this is not the first report killing of NK cells killing astrocytes, this is the first report exploring some of the mechanisms of how NK cells can kill astrocytes. We identified a novel ligand for NKp44 on astrocytes. Expression of this novel ligand decreased with increasing HIV 3S peptide concentration and blocking this novel ligand decreased NK cell killing. NK cell killing of

astrocytes was decreased when astrocytes were incubated with HIV 3S peptide. Our study also found an increase of CD38 expression on primary NK cells after incubation with astrocytes and NK cells were able to lyse astrocytes through CD38 signaling. This suggests that NK cells migrating to the CNS after brain injury can lyse healthy astrocytes via CD38 interactions resulting in neurotoxic effects. However, during the course of HIV-1 infection of the CNS, NKp44L is decreased and leads to a protection of astrocytes from NK cell killing, suggesting a viral escape mechanism from NK cell mediated killing (Figure 5.1). This study proposes that NK cells can initiate neurotoxicity or neuroprotection during HIV-1 infection of the CNS and could play a role in HAND.

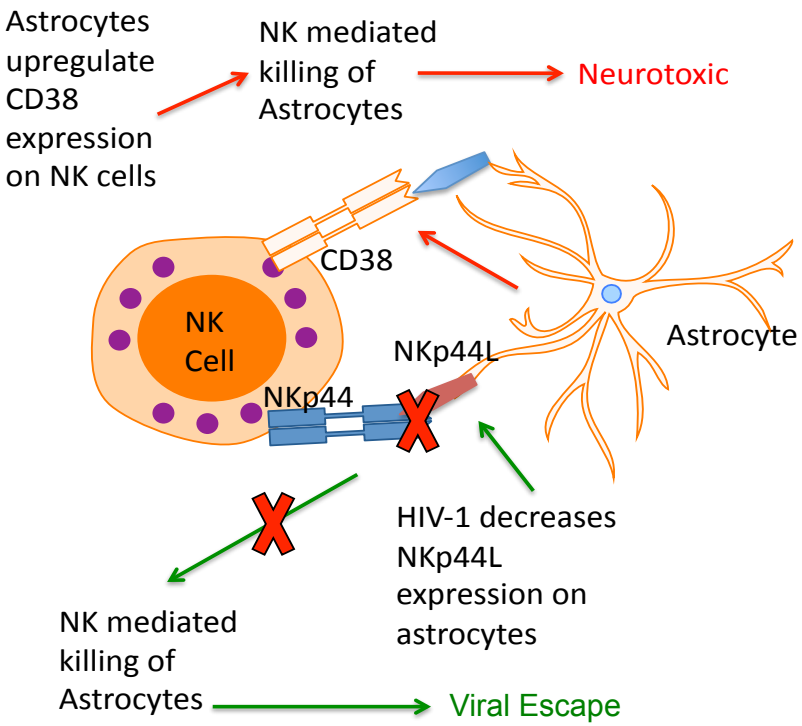

Figure. 5.1 NK cell interaction with astrocytes could be toxic or allow for viral escape from NK cell mediated killing in the context of HIV-1 infection of the CNS. NK cell interaction with healthy astrocytes could increase CD38 expression on NK cells, making astrocytes more susceptible to NK cell mediated lysis, suggesting a neurotoxic effect. On the other hand, in the context of HIV-1 infection of the CNS, astrocytes decrease NKp44L expression resulting in protection from NK cell killing.

5.4 Future Directions

NK cells mechanism of action during brain injury is still vastly understood. Our data sheds light on the role of NK cells in the CNS and is a stepping stone to future studies on NK-astrocyte interactions. This study is the first report of NKp44L on healthy cells. Blocking NKp44 interactions, decreased NK cell activity, suggesting NKp44L is an activating ligand on astrocytes. MLL5 was recently identified as an activating ligand for NKp44 [70]. It will be worthwhile to determine if the novel ligand for NKp44 on astrocytes is MLL5. If astrocyte incubation with anti-MLL5 prior to fusion protein incubation results in a reduction of fusion protein binding, this could suggest that MLL5 is the ligand. NK cytotoxicity assays comparing the lysis of astrocytes with anti-MLL5 to astrocytes with fusion protein will also assist in verification of the ligand. It is also logical to evaluate NKp44L expression and function on other glial cells, microglia, and oligodendricytes. This could help to develop strategies to prevent NK cell lysis of CNS cells specifically through NKp44. NK cells comprise 10-20% of CNS infiltrates in EAE brains [76]. It will be beneficial to determine the percentage of NK cells in HAND brains, and from this percentage determine effector:target cell ratios that will be specially relevant during HAND and HAD.

Due to the dual nature of NK cells and cytokine release in the CNS, it is difficult to determine if NK-astrocyte interactions are harmful or beneficial. Future studies providing novel insights into the role of NK cells in the pathogenesis of HAND and other brain disorders might result in the development of NK cell based therapies for HAND or other brain pathologies. For example, preventing of NK cells from killing healthy astrocytes or using NK cells to specially target HIV-1 infected astrocytes could be one possible future approaches to treat patients of HAND.

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