NEUROTROPHIN-CHEMOKINE RECEPTOR INTERACTIONS ON MACROPHAGES REGULATE HIV NEUROPATHOGENESIS

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum of Neurobiology in the School of Medicine.

Chapel Hill
2015

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ABSTRACT

Kimberly Williams: Neurotrophin-chemokine receptor interactions on macrophages regulate HIV neuropathogenesis
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Invading perivascular macrophages and microglial cells play a pivotal role in the neuropathogenesis of Human Immunodeficiency Virus (HIV) associated neurological disorders. As with many neurodegenerative diseases, the activation of macrophages and microglia cells in the central nervous system (CNS) leads to the secretion of unknown neurotoxins and is thought to contribute heavily to neuronal damage. However, these cells are very dynamic with a wide range of functions including neuroprotection and repair. It has become increasingly clear that strategies to modulate specific phenotypes of these cells have substantial therapeutic potential. This thesis has identified a novel neurotoxic phenotype in human macrophages that extends beyond the classical M1 inflammatory or M2 anti-inflammatory activation categories often seen in the literature. Macrophage phenotypes that correlated with the secretion of neurotoxins assessed by measuring calcium dysregulation in neurons consisted of suppressed calcium spiking and the formation of polarized podosomes. We found that macrophages not possessing this neurotoxic phenotype showed increased ruffling of the membrane and increased calcium spikes. In neurodegenerative diseases, an imbalance in pro-neurotrophins versus mature neurotrophins have been observed, however the actions of pro-neurotrophins on macrophages have never been studied. We have shown that, similar to their opposing roles in neurons, proNGF signaling in macrophages stimulated

the neurotoxic phenotype while mature NGF created a more neuroprotective phenotype which was dependent on TrkA. The neurotoxic phenotype was also driven by HIV in a $p75^{NTR}$ and CXCR4 dependent manner. Neurotrophin signaling was able to modulate the HIV neurotoxic phenotype by exacerbating neurotoxin production (proNGF) or partially restoring the non-toxic phenotype (NGF). This thesis for the first time identified an interaction between the neurotrophin receptors $p75^{NTR}$ and TrkA and the HIV co-receptor CXCR4 through the use of co-immunoprecipitation techniques, which may be involved in the regulation of neurotoxic phenotypes. NGF co-stimulation with HIV increased CXCR4 phosphorylation and association with G-protein receptor kinase 2 (GRK2). This contrasted with the decrease in GRK2/CXCR4 complexes seen with HIV stimulation alone. Phosphorylated CXCR4 (pCXCR4) was found in overlapping domains with $p75^{NTR}$ and TrkA, which was downregulated by HIV. NGF was able to restore the $p75^{NTR}$/pCXCR4 co-localization and partially restored pCXCR4/TrkA co-localization. This data indicated that NGF suppressed the HIV neurotoxic phenotype by facilitating phosphorylation of the HIV co-receptor CXCR4 while proNGF enhanced HIV effects. Overall, these studies have identified novel effects of the neurotrophins on macrophages that may provide new therapeutic avenues for the control of toxic macrophage phenotypes seen in HIV associated neurocognitive disorders and other neuroinflammatory diseases.

Dedicated to:
My mom, who sacrificed so much so that I could reach my dreams.

ACKNOWLEDGEMENTS

I would first like to thank my mentor Dr. Rick Meeker. During the past 6 years, Rick has been a constant supporter of all of my endeavors. I have truly been fortunate to have hands on training from a great scientist while also being encouraged to be involved in other activities outside of the lab. Rick selfless personality shined through during his willingness to stay late to help me with grants and preparation for conferences. He has truly been a great collaborator on this dissertation and has also taught me many things outside of lab from baking to exposing me to new cultures. I cannot begin to thank Rick for everything he has done for me over the years.

In a lab for six years your only hope could be to enjoy the people that you must work with on a daily basis. I have been truly blessed with amazing labmates/friends. I must thank Deirdre Killebrew for training me in the beginning and constantly being of assistance throughout the years. I also would like to thank Youmei Xie and all of the undergraduates that have rotated throughout the lab. A special thank you to my undergraduate, Tori, for all of her help with the co-immunopercipitation experiments. This past year you have become a scientist destined for great things. Thank you Dierdre, Gillian and Jaimie for all your help with the flow studies, calcium studies and experimental advice. Also during this time I must say without Gillian Clary and Jaimie Seawell I would have been miserable. It is truly amazing how you are provided with the tools that you need in life. Both of these girls were constant supporters and cheerleaders of mine. They laughed, made up “you can do it” dances

and worked to the core with me when I needed it. I am so grateful that during this work experience I was able to acquire such great friends in the process.

PREFACE

To go through life and get through grad school, a strong community is necessary. Though these people did not have a direct impact on the studies in this dissertation they have helped shape me in to the woman I am today , for this I thank them.

I would like to that Dr. Pat Phelps. Without Pat, my life would be completely different today. I remember our first one on one conversation where Pat was honest and straight forward with me regarding my options for graduate school and what it would take to reach my career goals. Because of this conversation, I have vowed to be honest, available and straight forward with students that I come in contact with who share similar experiences and backgrounds as myself. One of the major lessons learned from Pat is to find opportunities that coincide with my personality and that will truly make me happy. I admire Pat's free spirit and her ability to try new endeavors (And master all of them). I aspire to be as fearless and resilient as Pat one day and am so glad to have her in my life.

Benjamin Philpot, my first mentor at UNC has taught me so much over the year. Because of him I now project and command a room much better than the girl that began in his lab in 2008. Throughout the years I have been able to really count on Ben for encouragement and support in all of my endeavors. He has always been a floor and email away when I needed him and I truly appreciate everything he has done to support me over the years.

During graduate school, you have some up days and you have some down days. Through it all having a good group of friends can make any situation that much greater.

Arriving at UNC, I had no friends or close family in the area. The people in the Philpot lab became my first friends. Rylan Larsen, Thorfinn Riday and Maile Henson truly made the transition much more enjoyable for myself. I knew throughout the years that I could always count on them for a good laugh, all of the neurobiology gossip and any late night help (Thorfinn). Maile in particular became a cool mother figure for me that taught me not only how to be a rock star western blotting queen but also how to stay grounded and true to my values. Throughout my scientific career I will never forget her words of advice, keep your friends that are outside of science close because they will help keep life into perspective and although many around you may not believe hold true to your own belief system because though we are researchers, God is only allowing us to see small pieces of the puzzle through our research.

I would also like to thank the many other friends I have made during my graduate career. Kenton Woodard and Reginald Cannady have been true backbones for me during my graduate career. Kenton and I took every class that I had at UNC. We studied together, we encouraged each other and have become lifelong friends. I know great thing will come from him in the future and I just cant wait to see it. This is also true for Reggie. If it was not for Reggie to prep and encourage me through the long check list that is graduate school, I would have been forced to figure it out on my own. His support and friendship has truly been a blessing during this time. Also my grad school experience was balanced thanks to a group of girls that I call my friends. Grace Silva, Samira Brooks and Tikvah Hayes have been a joy to get to know. We have been there to be a listening ear and the turn up crew when needed. I have enjoyed getting to know these girls and look forward to many more turn ups!

To my life long friends:

God assigns you some family members at birth and then allows you to think you are picking some throughout life. In my almost 29 years I have gained a family that are often times more present and supportive than my blood relatives and for that I am thankful. To my two childhood best friends, Breauna Moore and Ashley Phillips, you were the sisters that I didn't have from the age of 5. Even though we haven't been in constant communication over these last years, you two will always hold a special place in my heart. Thank you for everything from teaching me how to ride a bike to how to be a true friend so early in life. My high school best friends, Jennifer Lowe and Debrienne Robinson, words can not how much your love and support has meant to me over the years. Thank you for calling, encouraging and supporting me all these years. Debee thank you for joining me on conferences so that I wouldn't have to travel alone and always having my best interest at heart during scholastic endeavors and life events.

Courtney Taylor, thank you for always being just a drive away when I need you. I don't know what I would have done during those times where I needed to get away if it had not been for you. Our countless weekend trips were really necessary to balance the stresses of graduate school.

Valencia Mattison, I will always thank you. You are one of my most selfless friends and grad school would have sucked without you here. I don't know of anyone else that can say that they have a friend that would pick up and move to a foreign city with no family and just one friend just to support them through graduate school. I love you for always writing my schedule down in your calendar just to make sure that you knew when to say "you got this". We have gotten each other through some challenging times and I am so glad to have you and my beautiful Godson, Kingston Sincere in my life.

Shannan Tonette, we went through grad school together and I could not think of a better person to have gone through it with. Form our daily morning conversations to the many conferences that we attended together having you along for the ride has made this all the much better. I think one day you will forgive me but if it wasn't for grad school we would have never found our favorite city and acquired this love for beignets. I cant wait to meet my newest baby, Ms. Aadyn Payne!!

Curtis Cain, how far we have come and this is only just the beginning. You have been the one that I can always count on and I cannot begin to explain how appreciative I am to have you in my life. I knew at a very young age that you were something special and that as a team we are unstoppable. I can not wait to see what we are about to do next. I love you forever and always.

To my family, thank you for all of your love support and prayers.

To my grandmother, I know that you are proud of me and have been rooting me on and probably helping out along the way. I miss you and love you everyday.I hope that I continue to make you proud.

To my brother, Brandon, the sky is the limit and I can wait to see you reach it. You have so much potential and I hope that I have been your encouragement to take on this world and be the best Brandon that you can be. I love you more than you may ever realize and I am glad God gave me you!

Mommy.

Thank you for loving me and being there for me unconditionally my entire life. Your sacrifice is the reason that I have made it thus far. You have taught me to be strong and to go

after my dreams. I have learned so much from you that has allowed me to be a better person. I can never express how appreciative I am to have you as my mother and I am so glad that God chose me for you. We together are now doctors. It is time for you to start living your life again for you because we made it and its all thanks to you! I love you to the moon and back!!

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LIST OF ABBREVIATIONS

ABI 7500	Applied biosystems (PCR system)
aCSF	artificial cerebrospinal fluid
ADC	AIDS Dementia Complex
AIDS	Acquired Immune Deficiency Syndrome
ANI	asymptomatic Neurocognitive Impairment
ANOVA	Analysis of variance
ART	Antiretroviral Therapy
B27	Gibco B-27 supplement
BCA	Bicinchoninic acid
BDNF	Brain derived neurotrophic factor
BSA	Bovine serum albumin
CART	Combined Antiretroviral therapy
CCR5	C-C Chemokine Receptor type 5
CD	Cluster of Differentiation
CD4	Cluster of Differentiation 4
CNS	Central Nervous System
CRAC	Calcium release activated channel
CSF	Cerebrospinal fluid
CXCR4	C-X-C Chemokine Receptor type 4
DAVID	**D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery

DMEM	Dulbecco's modified Eagle medium
FBS	Fetal Bovine Serum
FGF	Fibroblast growth factor
GADPH	glyceraldehyde 3-phosphate dehydrogenase
GDF	Growth differentiation factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
Gp120	Glycoprotein 120 (HIV envelope protein, 120 kDa)
GPCR	G-protein coupled receptor
GRK2	G-protein coupled receptor kinase 2
HAART	Highly active antiretroviral therapy
HAD	HIV Associated Dementia
HAND	HIV Associated Neurocognitive Disorder
HBSS	Hank's balanced salt solution
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HIV	Human Immunodeficiency Virus
hMDM	Human monocyte-derived macrophage
HRP	Horseradish peroxidase
IL	Interleukin
IP (10)	Interferon gamma-induced protein
LPS	Lipopolysaccharide
MCM	Macrophage conditioned medium
MEM	Modified Eagle medium
MIP	Macrophage inflammatory protein

MMP	Matrix metalloproteinase
MND	Minor neurocognitive Disorder
NGF	Nerve growth factor
NT-3	Neurotrophin 3
NT-4	Neurotrophin 4
NTR	Neurotrophin Receptor
$p75^{NTR}$	p75 neurotrophin receptor
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffered saline
pCXCR4	phosphorylated CXCR4
PMSF	Phenylmethylsulfonyl fluoride
proBDNF	pro Brain derived neurotrophic factor
proNGF	proNerve growth factor
RIPA	Radio-immunoprecipitation assay
SDF-1	Stromal Derived Factor-1
SDS	Sodium dodecyl sulfate
TGF	Transforming growth factor
TIMP-1	Tissue inhibitor of metalloprotease 1
TrkA	Tropomyosin Receptor Kinase A
TrkB	Tropomyosin Receptor Kinase B
TrkC	Tropomyosin Receptor Kinase C

CHAPTER 1: INTRODUCTION

Inflammation in the Central Nervous System

The central nervous system (CNS), consisting of the brain and spinal cord, was once thought to be an immune privileged environment protected from debris and systemic immune cell infiltration by the blood brain and blood cerebrospinal fluid barriers. However, we now know that there is constant communication between the immune system and the central nervous system to ensure proper homeostasis and signaling in the brain. The introduction of external stressors such as injury and infection can lead to inflammation in the brain known as neuroinflammation [1]. Short periods of neuroinflammation can be beneficial in the central nervous system, however, prolonged and excessive activation of these immune responses can cause chronic inflammation that leads to neuronal damage and loss[2]. Excessive inflammation can take many forms and is often seen in neurodegenerative diseases[3]. This thesis will focus on inflammation resulting from HIV infection that underlies the development of HIV Associated Neurocognitive Disorders (HAND). Understanding how to control inflammation in the central nervous system is important in creating new therapeutic avenues for HIV neuropathogenesis, and a better understanding of these processes is a major goal of this dissertation. The following section reviews the normal composition and functions of immune cells in the central nervous system, the major immune cells in the brain, microglia, and how they are activated to create chronic neuroinflammation.

Normal composition of Immune cells in the Central Nervous System

Under normal conditions, immune cell infiltration into the brain is highly restricted by the blood brain barrier resulting in a vastly different composition of immune cells inside the brain compared to other organs. For example, the population of T cells in the brain parenchyma is one tenth of its appearance in other organs. Immune surveillance in the brain is mediated primarily by microglial cells, also known as the resident macrophage of the brain. Microglia/macrophages are a part of the mononuclear phagocyte group and are among the first cells to respond to injury and infection during innate immunity [2,4]. Microglia represent 0.5%-16.6% of cells present in the brain and are found in higher quantities in white matter regions compared to grey matter regions [5]. Microglia cells are normally tightly controlled quiescent cells in the central nervous system with distinct roles in maintaining a healthy environment. These roles include surveillance of the microdomain, removal of debris and noxious agents through phagocytosis, modulation of synaptic connections and plasticity, communication with other brain cells and responding to infectious and toxic agents[6].

Astrocytes, the most abundant glial cells in the CNS, also contribute to innate immunity, though to a much lesser extent. Astrocytes are found throughout the brain in both white and grey matter regions. Under normal conditions astrocytes support neuronal function, secrete neurotrophic factors and aid in neural activity and homeostasis. Physically, astrocytes influence the formation and maintenance of the blood brain barrier by contributing to its structural and functional integrity[7]. While they are not classical immune cells, astrocytes respond to insult and injury by changing cell morphology resulting in cell hypertrophy and scar formation referred to as astrogliosis. Astrocytes are also able to secrete pro-inflammatory cytokines and complement proteins. Though astrogliosis has been associated with neurodegenerative diseases such as Alzheimer and Parkinson disease and

HIV associated cognitive disorders, it will not be a focus of this dissertation.

Immune surveillance in the CSF

Though immune cell populations are low within the parenchyma of the brain, constant surveillance and communication of the central nervous system is always occurring by systemic immune cells located in cerebrospinal fluid (CSF) and specialized structures just outside the CNS proper. Studies have shown that approximately 450,000 white blood cells are normally found within the CSF of healthy adults [8,9]. The composition of these cells is quite different from blood indicating that these cells are highly selected for entry into the brain compartment. Though there are reduced numbers of T cells in the brain, T cells can traffic into the CSF to survey and elicit signals to the central nervous system through interactions with ependymal and epithelial cells that line the ventricles and choroid plexus respectively[10]. Flow cytometry studies have shown that the T cells found in human and mouse CSF were CD4+, activated central memory (CD45RA-/CD27/28+) T cells, suggesting that they were recruited into the central nervous system, possibly through the choroid plexus or meninges[11]. T cells are thought to make the greatest contribution to HIV in the CSF, although the interactions of virus with cells in the parenchyma and the contributions to associated cognitive disorders are unclear.

The central nervous system is also surveyed by subsets of monocyte-derived macrophages, which include perivascular macrophages, choroid plexus macrophages and meningeal dendritic cells. These specialized macrophages usually remain in their respective locations under normal conditions relaying systemic information to the CNS and receiving messages from the brain[12]. However, it is controversial whether these macrophages enter the brain parenchyma under special conditions such as to replenish the long lived microglia

during aging and in response to adverse conditions like infection. Varvel et al. has shown that after experimental depletion of microglia from mice, the microglia were replenished by engrafted peripheral blood derived monocytes entering the brain from small blood vessels and capillaries[13]. Similarly in a Simian Immunodeficiency Virus (SIV) animal model for neuroAIDS, labeled monocytes in bone marrow were able to travel into brain tissues more readily than in uninfected macaques [14]. Other studies, however, have tried to determine if the expansion of microglia in the parenchyma of the brain during chronic and neurodegenerative disease were from infiltrating perivascular macrophages resembling microglia or proliferating microglial cells [15,16] and determined that the populations represented a microglia expansion.

Macrophage/Microglia activation and its role in Neuroinflammation

Neuroinflammation has been widely implicated as a pivotal event contributing to neurodegeneration. Though many neurodegenerative diseases have different causative etiologies, there are common pathologies seen among these diseases. Of importance, macrophage activation, permeability of the blood brain barrier, infiltration of systemic macrophages and secretion of neurotoxins have been noted in neurodegenerative diseases such as Alzheimer disease and HIV associated dementia (HAD)[17-20]. Though the mechanisms for these common pathologies are not fully understood, it is well accepted that macrophage activation is a key step.

Macrophages/Microglia cells are highly dynamic and can express a wide range of phenotypes driven by their ability to respond to a wide range of external cues. Activation by these environmental stimuli occurs through interactions with many cell surface receptors that

mediate functional changes in macrophages including morphology, migration, phagocytosis, cytokine secretion and antigen presentation. Though it was thought that macrophages were only able to produce pro-inflammatory factors, being one of the most secretory cells in the body, macrophages are capable of releasing factors needed in homeostasis, inflammation and host defense responses under the control of multiple mediators. Macrophage activation phenotypes are transient and reversible depending on cues received from the microenvironment and range from strong inflammatory responses designed for elimination of invading pathogens to anti-inflammatory, protective and wound healing activities essential for tissue repair. This versatility in functions has led to the characterization of two polarized activation states, classical activation or M1 and alternative activation or M2 [21-23]. Reports have suggested that macrophages may require at least two signals to achieve polarization [24].

Classically activated inflammatory macrophages have been shown to arise from type 1 cytokines such as interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and lipopolysaccharide (LPS) stimulation. M1 induction can also occur after recognition of pathogen-associated molecular patterns (PAMPS) and Damage- associated molecular patterns (DAMPS) on foreign or damaged agents such as viruses, bacteria and dead cells. M1 macrophages also express increased MHC class I and II receptors and secrete complement factors necessary for complement-mediated phagocytosis[25]. Activation of M1 macrophages leads to secretion of pro-inflammatory cytokines and chemokines such as IL-12, IL-23, TNF-α, IL-1, and IL-6 and production of reactive oxygen species which damage proteins.

Alternative M2 activation of macrophages is the balancing anti-inflammatory

activation state of macrophages leading to the secretion of anti-inflammatory cytokines, chemokines, growth factors and other reparative factors[26]. M2 has been sub-divided by some into 3 groups M2a, M2b and M2c [23]. M2a is induced by IL-4 and IL-13, while M2b is induced by immune complexes in combination with IL-1β or LPS and M2c is induced by IL-10, TGF-β or glucocorticoids. These activation categories however are overly simplistic and often a hurdle to understanding the wide range of phenotypes of macrophages, as many studies fail to go beyond a characterization of the classical cytokines. Hyperresponsiveness of classically activated macrophages can lead to tissue damage while exaggerated wound healing properties have been shown to increase infections in severe burn patients indicating that a balance of activities must be maintained. Understanding how these activation states may intertwine to create the proper balance in macrophage function is important for the development of precisely targeted macrophage therapies in the future.

In the normal CNS, microglia are in a resting homeostatic state and activation is tightly controlled to avoid potential indirect damage to neurons, which cannot readily regenerate and repair. To achieve this, neurons aid in controlling the resting state of microglial cells through constant communication carried out by specialized cell surface receptors and their ligands on both cells. Specifically, signaling partners, CD200/CD200 receptor and fractalkine /CX3CR1 have been implicated in controlling the resting state of microglia[27]. Loss or deficits in these receptors has resulted in increased microglial activation in animal models and have been implicated in neurodegenerative diseases[28]. The resting state can also be compromised from external stressors such as HIV infection of the brain, leading to chronic activation of microglial cells and subsequent neurodegeneration. The activated cells responding to HIV infiltration into the brain portray many of the classical

M1 phenotypes such as increased secretion of proinflammatory factors and upregulation of the cell surface marker, CD16[8,17,29-31]. This inflammatory activation is associated with neuronal damage and a gradual progressive degeneration of the CNS without appreciable cell death. This suggests that damage caused by microglial/macrophage activation may be reversible and interventions that restore the balance of microglia activation states may be fruitful therapeutic options for HIV associated disorders. This thesis aims to identify novel signaling interactions capable of establishing favorable macrophage phenotypes that prevent HIV neuropathogenesis.

General overview of HIV infection

In order to understand how to control the HIV-associated inflammatory cascade responsible for HIV associated cognitive disorders, it is important to understand the general nature of the virus and how it infects and interacts with its host cells.

History of HIV: a public health problem

Human immunodeficiency virus (HIV) is an epidemic affecting an estimated 1.2 million Americans in the United States and is predicted to have 50,000 new cases each year (http://www.cdc.gov/hiv/statistics/basics/ataglance.html). HIV is transmitted through bodily fluids such as mucosal barriers and blood. The most common form of transmission is through sexual transmission, and HIV is the number one deadliest sexually transmitted disease. The first cases of HIV described in the 1980's were 5 homosexual men presenting with *Pneumocystis carinii* pneumonia (PCP). HIV was originally thought to be a disease affecting only this population as more reports of homosexual men diagnosed with Kaposi Sarcoma followed. However, non-homosexual, intravenous drug users and blood transfusion recipients

began to surface, negating these theories [32,33]. HIV quickly became an epidemic because we now know the early stages of disease present with mild symptoms, resulting in the transmission of virus from patients unaware that they were infected. To this day, 1 in 7 people infected with HIV are unaware of their status, which contributes to the continuous rise in infection. Though patients presented with various opportunistic infections, they all shared one common hallmark, depletion in CD4+ T cell counts that led to a decline in the patient's immune system. In 1983 HIV was discovered as the virus that caused Acquired Immune Deficiency Syndrome or AIDS [34,35].

HIV Infection

HIV infection can be broken down into four stages: primary acute infection, a clinically asymptomatic stage, symptomatic HIV infection and progression from HIV to AIDS. After transmission, primary infection typically lasts 2 weeks, allowing virus to establish itself in the host body. During acute infection viral replication takes off, reaching peaks of millions of virus copies per millimeter of plasma just 21-28 days after infection, and CD4+T cell numbers begin to decline. Like in the blood, viral loads in the CSF can peak around week three, though found at ~1% of the quantities in the blood. Patients have flu-like symptoms 90% of the time during acute infection, and signs of meningoencephalitis may occur. Clinical symptoms at this point do not warrant suspicion of infection unless a history of HIV risk is known as antibodies against HIV cannot be detected in the host blood until 6-12 weeks post infection. Before the second phase of infection, there is a rapid drop in viral production and slight recovery of T cells. In the second phase known as the asymptomatic phase, HIV continues to replicate and a gradual decline of T cells occurs leading to symptomatic HIV infection and AIDS. Neurological deficits are the first signs of

symptomatic infection in 10-20% of patients. With severe HIV infection this increases to 60% of patients with clinically relevant CNS manifestation. Approximately 30-40% showed signs of dementia. Once CD4+ T cell counts fall below 200 cells per milliliter, patients are diagnosed with AIDS. During the AIDS stage, patients are more likely to suffer from opportunistic infections and severe dementia, which leads to their demise.

Currently there is no cure for HIV and the current treatments include several classes of antiviral drugs used to block the main steps in replication: entry, fusion, reverse transcription, proteolytic processing and integration. The first antiretroviral treatment was approved by the FDA in 1987, a nucleoside reverse transcriptase inhibitor, azidothymidine (AZT, Zidovudine). This drug was ineffective in suppressing long term viral replication. Since then it has been discovered that a combination of at least three antiretroviral therapies used to block different phases of HIV replication is needed to manage infection. This strategy became known as highly active antiretroviral therapy (HAART), currently known as combination antiretroviral therapy (CART)[36]. The use of these drugs has led to longer life expectancy as patients have suppressed viral loads systemically and increased CD4+ T cell counts. However these drugs cannot effectively cross into the brain, where virus can persist. This poses a need for neuroprotective therapies in conjunction with standard CARTs. In order to achieve this we must first understand the nature of the virus and its interaction with the central nervous system.

Structure of the HIV Virion

HIV, a member of the lentivirus subgroup of retroviruses, is a round virion (100nm in diameter) containing a double stranded RNA genome in its core. HIV has an outer membrane known as the viral envelope consisting of a lipid bilayer containing viral proteins

necessary for fusion and entry into the host cell. The envelope protein which mediates infection is a 160 kDa glycoprotein known as gp160. The gp160 protein is cleaved and further glycosylation occurs in the Golgi to produce a 41 kDa transmembrane protein, gp41, and a 120 kDa surface envelope protein, gp120. Gp41 serves as the stalk for gp120. The gp41/gp120 complex forms oligomers of three molecules in the membrane and is necessary for virus entry into the host cell[37].

The viral core contains the double stranded RNA and proteins necessary for replication and integration into the host genome. The HIV genome consists of 9 genes: the structural genes that encode cell surface proteins: Gag, Pol, and Env; the regulatory genes that control replication: Tat and Rev; and the accessory genes involved in virus replication and continued infection: Vpu, Vpr, Vif, and Nef. The core also contains reverse transcriptase, integrases, and proteases which are encoded by the pol gene and are necessary for viral integration into the host genome[38].

HIV binding and entry into host cell

Viral fusion and entry into the host cell is dependent on the interaction of the viral surface glycoproteins with two classes of surface receptors on the host cell, the primary receptor CD4 and the co-receptors, CXCR4 and CCR5. CD4 is a transmembrane protein consisting of 4 glycoprotein domains (D1-D4) that are in high abundance on T cells. Macrophages also express CD4 though in much lower quantities. CD4 expression is necessary for proper infection of the host cell though it has no known functions under normal conditions. The co-receptors CXCR4 and CCR5 are chemokine, G protein coupled receptors (gpcr) that respond not only to HIV but also their natural ligands SDF (CXCR4), MIP-1□ and Rantes (CCR5) to orchestrate migration under normal conditions. HIV virions have

tropism preferences for the co-receptors, which explain the ability of some viral strains to infect T cells (CXCR4 preferring) whereas other strains infect cells of monocyte lineage (CCR5 preferring). Although the strong infection preferences under some circumstances suggested that the preferences might be mutually exclusive, it is now recognized that both chemokine receptors are expressed on each cell type and viral infection by T cell tropic or macrophage tropic strains is not exclusive to their preferred receptors. In addition, there are also dual tropic viruses, CXCR4/CCR5 that can infect through both receptors. The efficiency of entry is determined to a large degree by the abundance of CD4 in conjunction with either co-receptor[39].

HIV entry is achieved when the HIV surface protein, gp120 binds to CD4 causing a conformational change in the gp120 trimer allowing interactions with co-receptors CCR5 or CXCR4. Once gp120 binds to the co-receptor, a second conformational change occurs in gp41, exposing a fusion domain that embeds itself into the cell membrane resulting in fusion and the formation of a pore for viral entry into the host cell. Since most infection involves transmission at mucosal and cellular barriers, initial infection is typically mediated via the infection of CCR5 expressing macrophages that are abundant at these sites. The importance of CCR5 for entry and infection is evident in a select group of individuals who are resistant to infection (approximately 10% of Caucasians) due to a deletion mutation in the CCR5 gene on allele 32 (CCR5Δ32) that restricts the binding of CCR5 preferring HIV virions[40,41].

Integration into the genome and viral replication

After fusion occurs, the virion releases its material into the host cell during a process referred to as uncoating which releases the viral genome and proteins necessary for reverse transcription and integration. The initial replication steps occur in the cytoplasm of the host

cell and begin with reverse transcription of the viral double stranded RNA into double stranded DNA and formation of the replication complex. The replication complex also known as the pre-integration complex consists of the viral DNA/RNA, reverse transcriptase, integrase, viral matrix protein p17, viral proteases Vpr, histones and non-histone proteins. This complex is transported to the nucleus to begin integration. Once into the nucleus, the viral and host DNA is prepped for viral DNA insertion, catalyzed by integrases followed by ligation. Once integrated into the host genome the provirus begins to multiply, making viral RNA and mRNA that are later translated into viral proteins. After transcription of the viral copies, the RNA is transported out of the nucleus and the viral proteins and genomic RNA are then packaged in the cytoplasm of the host cell. Budding of the viral RNA and protein package is the final stage of virus production and occurs through exocytosis[42].

Replication and mutations

During heterosexual transmission, 80% of infection is initiated with a single variant[43]. On average, an infected individual will produce 10^{10} virions in a day[44]. Unlike mammalian cells, viral DNA repair is inefficient. This leads to a high mutation rate of 3 X 10^5 nucleotide bases per cycle of replication leading to the generation of many variants[45,46]. Over time the viral populations in a patient becomes increasingly more diverse contributing to the genetic variability of virions. This evolution of the virus aids in its long-term survival within the host and leads to resistance of HIV drug treatments causing the need for constant changes in a patient's therapy[47].

In the central nervous system, HIV replication is slow and the amount of virus synthesized within the CNS is still controversial. Phylogenetic analysis of viral envelope sequences in blood and CSF has shown that most virions match sequences from blood[48].

However, these analyses have also identified small groups of unique variants suggesting an evolution of viral sequences unique to the CNS[49]. This has led to the idea that strains of HIV compartmentalized in the CNS may have unique properties that encourage neurodegeneration[50,51] although this is still controversial. Understanding the interactions of HIV with cells in the CNS is essential for the development of strategies that control replication and disease progression.

HIV in the CNS

HIV infection in the CNS is a persistent problem that has evolved with the introduction and evolution of antiretroviral therapy. In this section we will review how HIV infection is thought to cause neuropathogenesis.

HIV in the CNS before the era of HAART

Before the introduction of HAART, a large proportion of patients presented with opportunistic central nervous system infections and neurological deficits ranging from mild cognitive-motor impairment to severe dementia. In 1987, patients with severe sub-acute encephalitis were identified as having AIDS dementia complex (ADC), currently termed HIV associated dementia or HAD[52-54]. HAD was characterized as subcortical damage causing severe cognitive impairment, altered psychomotor movements and severe disturbance in day to day activities [55]. Before the HAART era, 20-30% of patients were diagnosed with HAD [56,57] and it is unclear why only a subset of patients have clinically significant CNS impairment.

After the era of HAART

After the initiation of HAART treatment, the prevalence of HAD decreased drastically[58-60]. However 18-50% of HIV patients still present with milder neurocognitive disorders, now designated HIV associated neurocognitive disorders (HAND)[61]. HAND encompasses HAD, asymptomatic neurocognitive impairment (ANI) and minor neurocognitive disorder (MND). ANI affects 24% of HIV patients with abnormal neuropsychological performance but no change in life activities while MND affects 52% of patients and also results in abnormal neuropsychological testing with mild interference in daily activities[62]. This reduction in clinical neurological symptoms suggested that neuronal damage created by HIV infection may be fully reversible and highly amenable to therapeutic intervention.

Target cells in the central nervous system

Though neuronal damage is responsible for the clinical symptoms, HIV does not infect neurons. Unlike systemic infection, the role of CD4+ T cells in the spread of HIV in the central nervous system is not clear. HIV infection of astrocytes has been reported in 1-3% of astrocytes [63] although this is not a common observation. Astrocytes lack expression of the primary HIV receptor CD4 and are unable to transcribe viral material[64] and thus do not produce infectious virions. Full length HIV genomes have not been detected in these cells, but multiple fragments of HIV RNA and proteins have been identified within astrocytes which may be released and have indirect neurotoxic effects[65]. The best supported contribution of astrocytes to HIV neuropathogenesis is via a loss of glutamate transporter function which is thought to facilitate excitotoxic damage [66]. Another potential indirect contribution of astrocytes to neuropathogenesis is through interactions with HIV soluble

proteins, nef and gp120, that lead to disruptions in the blood brain barrier increasing its permeability[67,68].

The main cellular targets that carry the HIV burden in the central nervous system are the perivascular macrophages and microglial cells. These long lived cells are able to harbor virus and produce a slow steady viral replication once virus enters the brain[69].

HIV entry into the central nervous system

HIV enters and infects the central nervous system shortly after the initiation of primary infection. Once infection is established the brain acts as a protective reservoir for HIV [70]. In human studies, HIV RNA has been measured in the CSF prior to treatment and during HAART treatment even when systemic infection is suppressed[71]. This phenomenon has been termed CNS escape. Though HIV entry into the CNS has been accepted since the 1980's, the mode of entry is still controversial today. The most accepted theory of HIV entry is the Trojan Horse hypothesis: Activated monocytes expressing CD14+/CD16+ cell surface markers, some of which are infected with HIV, are able to cross the blood brain barrier[29]. Once in the brain, monocytes are differentiated into perivascular macrophages. These activated macrophages are then able to produce more HIV, which then infects resident microglial cells.

Other theories suggest that cell free virus may diffuse across a disrupted blood brain barrier. However, these studies are largely based on findings from late stage, infection and it is not clear if there is disruption of the BBB during the initial stages of infection when the CNS reservoir is thought to be established. Soluble HIV surface protein gp120 has been implicated in disrupting the integrity of the blood brain barrier and enhancing monocyte migration[72]. Also it has been postulated that virus can interact with the endothelial cells

that line the blood brain barrier and modify proteins that are essential for maintaining tight junctions in the blood brain barrier[68,73].

Another mode of entry involves HIV entry via the choroid plexus. The choroid plexus is a unique organ with specialized functions including the production of cerebrospinal fluid (CSF). This intricate structure extends from the ependymal of the lateral, third and fourth ventricles of the brain and is the home of many immune cells including a large population of macrophages that survey the central nervous system, providing immune surveillance within the cerebral ventricles. HIV titers within the CSF can reach levels of 10^6-10^7virions /ml yet no study has shown that HIV or infected macrophages can enter into the parenchyma of the brain via the choroid plexus and ventricular system.

Mechanisms of Neuropathogenesis

The first clinical reports of neurological disorders during HIV infection showed remarkable neuronal damage and loss in post mortem analysis of patients with HAD symptoms compared to HIV patients with no signs of cognitive deficits[74,75] . The basal ganglia and hippocampus, regions that mediate cognitive motor processing and cognition, respectively typically show the most pathology leading to the description of HAD as a subcortical dementia. Other pathological features of CNS infection with HIV included infiltration of macrophages into the perivascular regions often presenting as multinucleated giant cells, clusters of activated microglia (microglial nodules), and a relatively diffuse microgliosis, and reactive astrogliosis [76-78]. Of all the pathology seen, activated macrophages are the best correlate for neurocognitive disease severity [79].

To date there has been no good indication of HIV infection of neurons although they do express co-receptors CXCR4 and CCR5 [80,81]. Some early studies showed that the neuronal damage was caused by the HIV surface protein gp120 which is cleaved from macrophages after engagement with the CD4 receptor [82,83]. Others have linked damage to cytokines such as TNF, arachidonic acid, quinolinic acid and other HIV proteins including Tat and Nef [84,85]. Of all of these factors the greatest support for a pathogenic role has been for gp120 and Tat. Various direct and indirect neurotoxic mechanisms have been hypothesized but a common theme has been the ability of the proteins to induce an inflammatory response. This, in turn, has led to many studies showing that soluble proteins secreted by stimulated macrophages produced neural damage. Lynn Pulliam and Dana Giulian were the first to show that HIV infected macrophages secreted neurotoxic factors leading to neuronal damage [17,18]. Lipton showed subsequently that the secretion of neurotoxins from microglia does not require infection and that the gp120 surface protein was sufficient to induce the secretion of these factors[86]. In a feline immunodeficiency virus (FIV) model, both infectious FIV and its envelope protein FIV-PPR produce comparable levels of neurotoxicity [87]. Similar to human pathology, gp120 transgenic mouse brains have increased appearance of vacuolizations in dendrites, decrease in synapto-dendritic complexity and increased activation of macrophages. With this appreciation that macrophage secretion of neurotoxins is the culprit for neuronal damage, the search for the neurotoxic factors has been an ongoing struggle[88] yet to be resolved.

These initial studies and observations have led to the ability to study neuropathogenesis in culture. Cultured neurons in the presence of challenged macrophages/microglia or their conditioned medium can allow cellular analysis of neuronal

damage as it evolves during early stages of pathogenesis. Hallmarks of early neuronal damage include swelling of the soma, calcium dysregulation, and beading along dendritic processes [89,90]. Although neuronal death and apoptosis have been documented and has been highly investigated as a mechanism of neuronal death, it represents a very small number of cells seen in the late stages of infection. Indeed the cognitive symptoms in HAD can be dramatically reversed by antiretroviral therapy[60]. Thus, there is little evidence that neuronal death plays a significant role in the development of dementia except perhaps at very late stages of disease.

Alteration in calcium homeostasis has been implicated in neurodegenerative diseases such as Alzheimer, Parkinson disease and HAD and is known as the "calcium hypothesis for neuronal disease"[91]. Although many different definitions of neuronal calcium dysregulation have been used in different studies, studies in this thesis focus on the influx of calcium that occurs in two phases leading to neuronal damage and in extreme cases death. This bi-phasic calcium deregulation was originally worked out in cultured embryonic spinal neurons challenged with glutamate during excitotoxicity [92]. Neurons responding to macrophage derived factors show a similar calcium profile. The first phase is an acute influx in calcium initiated by the conditioned medium from activated macrophages. This acute influx in calcium lasts no more than two minute before returning to baseline calcium levels with a plateau developing at variable intermediate levels. In the second phase known as the delayed or pathologic phase, calcium influx rises and is sustained until the death of the cell or cells are returned to favorable conditions. Once neurons are returned to favorable conditions such as normal culture conditions or addition of neurotrophin ligands, neuronal integrity is

returned suggesting the potential role to reverse neurological dysfunction during HIV infection with proper therapeutic avenues.

During the delayed calcium rise, another hallmark of neuronal damage appears: the formation of beading along the dendritic processes. This beading can also be seen in post mortem tissue from HIV patients with HAD. Beading is dependent on calcium, [93] and in an FIV model it has been shown to follow the delayed calcium influx in neurons [89].

Neuroprotection

Mechanisms of neuroprotection and animal models

Though the initiation of HAART has reduced the prevalence of HIV associated dementia in patients, there is still a need for neuroprotection as many patients still present with mild to moderate cases of neurocognitive impairment[94] . It is also predicted that as patients age, HIV cognitive disorders will continue to increase in prevalence and severity. When assessing how to therapeutically target HIV in the central nervous system three general strategies have been followed: 1) complete eradication of the virus from the central nervous system, 2) blockade of neuronal damage and 3) suppression of macrophage inflammatory activation.

Eradication:

Eradication of the virus from the central nervous system is a hard task because the brain is a protected reservoir that harbors virus that is unique to this compartment. Strategies for viral eradication of systemic latent T cells are currently ongoing [95] but are more difficult to apply to macrophages and microglia. Because HIV is integrated into the cell

genome, eradication strategies would have to reactivate viral replication in order to identify and kill infected cells. It is unclear if there is latent virus in the CNS or a low persistent level of replication. In either case difficulties in identifying and eradicating latent virus or destroying the resident immune cells hosting virus would be technically very challenging and potentially more damaging to the CNS than the infection. A better understanding of HIV in the central nervous system and how virus is harbored will be necessary in order to make complete eradication of virus from the brain a possibility[96].

Inhibiting Neuronal Damage:

Though the mechanisms of neuronal damage are not fully understood, calcium dysregulation via suppressed clearance processes in conjunction with activity of NMDA glutamate receptors or voltage gated calcium channels, oxidative stress and structural damage have been implicated as primary underlying causes. Neuroprotective therapies that cross the blood brain barrier and target these processes have been explored. Some have gone to clinical trials but none have yet proven to be effective.

One potential therapy that has been tested in animal models of HIV infection and in clinical trials was a non-competitive NDMA antagonist, memantine. A severe combined immunodeficiency (SCID) mouse injected with HIV infected human monocyte-derived macrophages into the caudate and putamen was used to produce HIV encephalitis (HIVE) and resulted in impaired synaptic transmission and long term potentiation in the CA1 region of hippocampus slices. Treatment with memantine significantly improved synaptic transmission in these mice. Simian immunodeficiency virus (SIV) infected macaques presented with a cocktail of viruses, showing accelerated signs of HIV neurocognitive

impairments and allowing researchers the ability to study progression of CNS degeneration over a few months. Greater than 90% of macaques develop SIV encephalitis when infected with two different viruses, a biological viral strain and a neurovirulent recombinant clone[97]. Memantine treatment administered to SIV infected rhesus macaques 2 weeks post infection, at peak viremia, prevented dopamine deficits often seen in this animal model. Memantine also upregulated mRNA and protein expression of brain derived neurotrophic factor (BDNF), suggesting a potential role in neurotrophic support [98]. Based on the beneficial effects seen in animal studies, memantine was tried in a phase 2 clinical trial to treat HIV neurocognitive disorders; however, subjects showed no significant improvement in neuropsychological performance[99]. One promising neuroprotective modulator that is currently in clinical trials to treat Alzheimer disease, LM11A-31, has been a focus in our lab for the treatment of HIV associated dementia. In these studies, a naturally occurring feline immunodeficiency virus (FIV) animal model recapitulates disease progression in HIV infected patients. FIV rapidly penetrates the CNS and leads to neuropathogenesis. Neuronal damage in this model is similar to what is seen in HIV infected patients albeit less severe. In mixed neural cultures (neurons, astrocytes and microglia), FIV induced calcium dysregulation followed by the formation of varicosities along processes. Pretreatment of feline neurons with LM11A-31 blocked neuronal damage normally seen after treatment with conditioned medium from FIV-infected choroid plexus macrophages. This provided strong in vitro evidence that neurotrophic support may be a new potential therapeutic avenue for neuroprotection in HAND and other neuroinflammatory disorders [89].

Suppression of macrophage activation:

Much attention has begun to surface around the regulation of macrophage and microglial activation during disease. Though there is much that still needs to be understood about HIV induced macrophage activation, it has been shown that much of the toxic activity can be attributed to non-infectious interactions. HIV surface glycoprotein, gp120, can activate macrophages through the G-protein coupled co-receptors CXCR4 and CCR5, independently of binding to the primary HIV receptor CD4[86,100]. The relative role of CCR5 or CXCR4 tropic viruses in the generation of neural damage has been actively debated in attempts to determine if particular HIV strains have greater neurovirulence. HIV virions in the central nervous system are typically CCR5 tropic, however, macrophages also contain CXCR4 receptors. Studies have shown that both CCR5 and CXCR4 tropic virions result in the secretion of toxins by macrophages however the exact mechanism is not clear [101]. HIV engagement with these receptors activates downstream MAPK signaling (Erk1/2, JNK, p38) and phosphorylation of pyk2 and FAK which have been implicated in morphological changes of the cell leading to migration and secretion of cytokines and other neurotoxic factors[102,103].

Studies have reported that HIV gp120 increases macrophage secretion of M1 markers such as TNF-α [104-106] whereas others have shown that HIV infection increases the M2 marker IL-10[107-109]. These seeming discrepancies in the polarization of macrophage activation are difficult to reconcile unless one assumes that macrophage activation states reflect much more complex phenotypes.

While anti-inflammatory agents are routinely used for a wide variety of diseases, they typically suppress all activity globally or act through mechanisms that are not known. These

have failed to provide significant long-term neural protection in clinical trials for many neurodegenerative diseases. One in particular that was assessed for the treatment of HAND is minocycline. Minocycline, a tetracycline antibody, has been shown to suppress the expression of M1 markers with no transient effect on M2 markers [110] through mechanisms that are unclear. In culture minocycline inhibits microglial activation and reduces excessive matrix metalloproteinase activity and nitric oxide synthesis in neuron/microglial co-cultures from human fetal brain. Also, minocycline treated SIV infected macaques showed a reduction in perivascular macrophage infiltration and an overall reduction of CD68 and MHC class II expressing cells in the brain compared to untreated SIV infected animals. Minocycline decreased SIV encephalitis by reducing HIV viral loads in brain tissue and CSF, the inflammatory chemokine MCP-1 in CSF and axonal degeneration in this animal model. [111-113]. Infected macrophages in culture treated with minocycline showed reduced viral production and prevented reactivation of virus in latent infected cells[113]. A Phase II clinical trial was conducted for treatment of HIV associated cognitive impairments. Though hopeful, no improvement in neuropsychological test was seen at the end of this study[114,115].

Macrophages respond to many external cues depending on which tissues they are located in and the health of their environment. In the central nervous system neurotrophic support is necessary for neuronal health and stability. In the FIV model, increased microglial expansion and activation was seen in co-cultures with neurons. LM11A-31, the neuroprotective modulator that blocked neuronal damage induced by macrophage conditioned medium was also able to reduce the number of microglia seen in these cultures. In unpublished data LM11A-31 also blocked neurotoxin production from macrophages. This

raised the possibility that the neuroprotective effects of LM11A-31 could be linked to actions of the drug on macrophages as well as neurons[89]. However, very limited information on expression and function was available to determine if neurotrophin receptors were likely targets. Understanding how neurotrophic support in the central nervous system may alter macrophage activation during normal and pathologic conditions such as HAND is important for future therapeutic development, and is a primary goal of this dissertation.

Neurotrophin Signaling

As early as 1996 Levi-Montalcini [116] introduced the idea that neurotrophins may regulate the immune response. Since this early observation, a handful of studies have explored neurotrophin receptor gene transcript expression and immunoreactivity in immune cells in blood and various pathologic tissues[117]. These studies have shown that neurotrophin receptors are expressed on many types of immune cells. However few details were available to indicate the level of expression, localization and functions of the receptors. The presence of neurotrophin receptors on macrophages and microglia would provide considerable opportunity for neurotrophin regulation of immune cell function, particularly in the nervous system where neurotrophins are abundant. However, this aspect of neuroimmune interactions has received little attention and much of what we know regarding neurotrophin signaling comes from neuronal studies.

In the nervous system, neurotrophins and their receptors play a fundamental role in the development and maintenance of neurons. They have a wide range of well documented functions within both the central nervous system and peripheral nervous system (PNS) including established roles in neuronal differentiation, regulation of neurite outgrowth, synaptic regulation, cell survival and death [118]. Many of the functions are context and

tissue specific providing a highly versatile system for the development and maintenance of proper brain function[119-122]. The neurotrophin family is comprised of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). Each neurotrophin exerts unique actions via interactions with specific high affinity tropomyosin regulated kinase (Trk) receptors. NGF interacts with TrkA, BDNF with TrkB, NT-3 with TrkC and to a lesser extent with TrkA and TrkB and NT-4 with TrkB. In addition to the Trk receptors, both the mature neurotrophins and their pro-neurotrophin precursors can bind to the p75 neurotrophin receptor ($p75^{NTR}$) with low and high affinity, respectively[123]. Trk receptors and their ligands are involved in neuronal survival, differentiation, proliferation, migration and synapse formation. Binding of neurotrophins causes dimerization of Trk receptors and activation through auto phosphorylation. The $p75^{NTR}$ is a member of the tumor necrosis factor (TNF) receptor family and contains a death domain. Unlike the Trk receptors, $p75^{NTR}$ lacks a catalytic domain and exerts its functions by interacting with other receptors[118].

Neurotrophins are synthesized by glial cells and neurons [124,125{Huang, 2001 #11152]} as a pro-neurotrophin which is then proteolytically processed to the mature neurotrophin[126]. Initially, the pro-neurotrophins were thought to be inactive precursors of the active mature peptides. With the later recognition that the pro-neurotrophins are physiologically active came numerous studies showing that the pro-neurotrophins are ligands for the $p75^{NTR}$. It is now well recognized that neurotrophin signaling is dependent on the balance between mature vs. pro-neurotrophins as well as the nature of the target receptor complex. The $p75^{NTR}$ plays a pivotal role in the regulation of various pro-neurotrophin and mature neurotrophin functions and the potential significance of this role has been elevated by

recent recognition of upregulated $p75^{NTR}$ expression in response to injury and disease[117] . The availability of high affinity targets for pro versus mature neurotrophins is highly regulated by the interactions of $p75^{NTR}$ with its receptor partner. Heteromeric association of $p75^{NTR}$ with Trks enhances mature neurotrophin signaling by increasing the affinity of the neurotrophin for the receptor by as much as 100-fold to a K_D of approximately 10 pM, [127-129] thereby increasing cell survival, differentiation and growth. In contrast, the $p75^{NTR}$ -sortilin complex binds pro-neurotrophins with high affinity and activates pathways that lead to apoptotic cell death. The best studied of the pro-neurotrophins is proNGF. ProNGF is synthesized and secreted by a variety of different cell types providing a rich source of cell-cell interactions[130-133]. Cleavage of proNGF can take place intracellularly by convertases or extracellularly by the enzymes furin, plasmin and matrix metalloprotease-7 (MMP-7) [134,135]. ProNGF and proBDNF have both been shown to trigger a $p75^{NTR}$-dependent apoptotic cascade[136-140]. Confirmation of the role of each pro-neurotrophin was provided by demonstrating protection using specific antibodies to proNGF[137,141] or proBDNF [142]. The apoptotic cascade induced by proNGF and proBDNF was dependent on the interaction of $p75^{NTR}$ with the co-receptor sortilin since addition of a fusion molecule containing the extracellular domain of sortilin prevented the apoptotic effects of proBDNF. In $p75^{NTR}$ deficient mice, less cell death is seen after axotomy [143,144], seizures[141] or ligation by proNGF[136]. Similar effects are seen after sortilin knockout[145]. Activation of the $p75^{NTR}$/sortilin complex is thought to induce cell death through extended activation of c-Jun N-terminal protein kinase-3 (JNK-3) which in turn induces the synthesis of pro-apoptotic factors while also phosphorylating and inactivating anti-apoptotic factors[119].

The dichotomy between the actions of mature neurotrophins versus pro-neurotrophins has led to the hypothesis that an imbalance may facilitate the progression of many neurodegenerative diseases and contribute to natural decline in aging. The potential significance of proNGF signaling was further elevated by the observation that proNGF was the predominant form in human brain and was increased by two-fold in the parietal cortex of patients with Alzheimer disease [146]. A corresponding loss of TrkA in patients with Alzheimer disease[147,148] and preservation of $p75^{NTR}$ and sortilin expression [147,149] provided further support for a shift in pro versus mature neurotrophin signaling[150,151]. Excess pro-neurotrophin signaling has also been implicated in the pathology associated with other neurodegenerative conditions. BDNF expression has also been shown to be down regulated in the hippocampus of a gp120 transgenic mouse model with impaired neurogenesis and neurite outgrowth. The pro form of this neurotrophin proBDNF was increased in gp120 transgenic mice compared to control [152]. Direct administration of BDNF was shown to be neuroprotective and block gp120 mediated neurotoxicity [153-155]. Also the loss of proper neurotrophin processing has been seen during brain injury. For example, the loss of MMP-7 activity in response to kainic acid induced seizures[156] and in diabetic retinopathy[157] is thought to contribute to the disease pathology by reducing the conversion of proNGF to NGF. However, other studies in HIV infected patients have shown that increases in MMP-7 activity are associated with a decline in neurological function[158]. These studies indicate that interventions designed to restore a more favorable balance of mature neurotrophin-Trk protective signaling have the potential to slow neurodegenerative processes. No studies have yet looked at potential interactions of pro-neurotrophins with immune cells; particularly microglia and macrophages within the neurotrophin rich CNS.

Neurotrophin Receptors Expressed on Immune Cells

Though much of neurotrophin signaling has focused on neurons, a few studies have explored the expression of these neurotrophin receptors on immune cells. Much of the work has occurred in pathologic tissues where it was hard to distinguish which cells contained neurotrophin expression. Other studies examined gene transcripts but not protein. The following section reviews what is known regarding neurotrophin signaling in immune cells with a focus on the expression and function of neurotrophin receptors on macrophages/microglia.

Studies have shown that CD3+ T cells from blood, tonsil, thymus and inflamed muscle do not express the $p75^{NTR}$ [159-162] although they do express small amounts of TrkA and TrkB. In involuted thymus from myasthenia gravis patients 15% of T cells expressed TrkB receptors. This expression was reduced in hyperplastic and neoplastic tissues. Conflicting data suggests the expression of TrkB and $p75^{NTR}$ at negligible to moderate levels in CD20+ B cells[163,164]. Natural killer (NK) cells isolated from human blood also expressed $p75^{NTR}$, TrkA and sortilin intracellularly [160,165]. Stimulation of the NK cells with IL-2 or IL-12 increased $p75^{NTR}$ but not TrkA or sortilin expression by up to 10-fold. Stimulation of NK cells with proNGF induced cell death similar to what has been seen in neurons[160]. Also, $p75^{NTR}$ and TrkB have been documented in eosinophils isolated from blood of atopic dermatitis patients [166]. TrkB and $P75^{NTR}$ expression in eosinophils was increased about 5-fold and 2-fold, respectively, in lesional mast cells of atopic dermatitis patients compared to control patients. Stimulation of eosinophils from atopic dermatitis with BDNF inhibited apoptosis in a dose dependent manner[166]. Similarly, increased $p75^{NTR}$

expression was found in human mast cells isolated from skin lesions of atopic dermatitis patients [167]. These results suggest varying expression of neurotrophin receptors on immune cells.

Macrophages/microglia

Neurotrophin expression has been identified in cells of the monocytic lineage in various tissues and at varying levels. Nakajima et al. reported that 99% of microglia expressed all neurotrophin receptors although the amount of expression varied among cells. This abundance in neurotrophin expression has not been seen in other studies although almost all studies show positive expression of neurotrophin receptors. Low to moderate $p75^{NTR}$ expression was seen in macrophages in inflamed muscle tissue[162]. $P75^{NTR}$ expression was absent in macrophages in the thymus and tonsils but moderately positive in the red pulp of the spleen in normal lymphoid tissue. Conversely, TrkA was expressed weakly in tonsils, lymph nodes and thymus and absent in spleen tissue [168] suggesting that expression may be tissue or context specific. Microglia, the resident macrophages of the brain, showed weak $p75^{NTR}$ expression in white matter regions of normal brain but expression was increased in glial cell bodies and large patches of macrophages/microglia in white matter MS plaques, as well as in glioblastoma, ALS and HIV encephalopathy [169]. TrkA and $p75^{NTR}$ have also been detected in monocyte derived macrophages isolated from human peripheral blood mononuclear cells. In cultures of human monocyte-derived macrophages (hMDM) stimulation with LPS increased TrkA expression by four fold but had no effect on $p75^{NTR}$ expression. NGF deprivation reduced this induction of TrkA expression yet increased $p75^{NTR}$ expression. LPS stimulated macrophages induced apoptosis in only 6%

of cells. However the addition of NGF neutralizing antibody in the presence of LPS induced a five-fold increases in apoptosis [170]. Garaci et al. also found that HIV infected macrophages expressed 30% more TrkA compared to control cells[171]. This HIV induced increase in TrkA expression was significantly reduced during NGF starvation. Conversely, 15 % of HIV-infected macrophages were immunoreactive for $p75^{NTR}$ in culture. $P75^{NTR}$ expression was increased to 35 % with NGF starvation and was accompanied by an initiation of apoptosis in approximately 45 % of HIV- infected cultured macrophages [171]. In a similar fashion, $p75^{NTR}$ expression was moderate to strong in microglial-like cells positive for tal1b5 and CD68 surrounding blood vessels and neurons in frontal cortical dysplasia, glioneuronal tumors and dysplastic neuronal tissue[172]. There were no Trk receptors found in these samples suggesting a primary role for $p75^{NTR}$ in these disease states. Moderate to strong $p75^{NTR}$ expression was also found in microglia in hippocampal sclerosis samples from temporal lobe epilepsy patients[173]. Taken together, there is strong support for the presence of neurotrophin receptors on immune cells but the results of expression have been inconsistent and the functional role of these receptors rarely addressed.

Functional studies of neurotrophin receptors on macrophages

There is limited functional data available for the role of neurotrophins on macrophages. Conflicting data has suggested a role for NGF in HIV replication[171,174]. One of the few studies that explored the effects of NGF on macrophages reported that NGF stimulation increased CXCR4 expression on the cell surface of monocytes and macrophages in culture but did not alter CCR5 or CD4 expression. This regulation of CXCR4 was specific to NGF as other mature neurotrophins did not affect CXCR4 expression. Samah et. al. concluded that NGF stimulation increased CXCR4 expression to enhance macrophage

chemotaxis to its endogenous ligand SDF-1[175,176]. However understanding how NGF may be regulating CXCR4 expression has not been explored. While these studies provide a glimpse of the possible role of neurotrophin receptors on microglia and macrophages, a greater appreciation of macrophage-neurotrophin receptor involvement in normal and pathologic conditions is needed. [113]

In this thesis I compare, for the first time, the involvement of mature NGF and proNGF in determining the functional activation state of human monocyte derived macrophages (hMDM) under normal conditions and in the context of HIV stimulation. I hypothesize that the activation state of hMDM is differentially regulated by neurotrophin receptors $p75^{NTR}$ and TrkA via interactions with CXCR4. ProNGF signaling through p75 synergistically signals with CXCR4 resulting in increased phosphorylation of CXCR4 leading to an active state associated with the generation of neurotoxins. Conversely, NGF signaling through TrkA is hypothesized to induce a more protective macrophage phenotype largely by interfering with the phosphorylation of CXCR4.

CHAPTER 2: DIFFERENTIAL REGULATION OF MACROPHAGE PHENOTYPE BY MATURE AND PRO-NERVE GROWTH FACTOR

Introduction

Macrophages are dynamic cells that can express a wide range of phenotypes driven by external cues. The phenotypes range from strong inflammatory responses designed for elimination of invading pathogens to anti-inflammatory, protective and wound healing activities essential for tissue repair. To highlight the different functional states, many studies have focused on characterizing the phenotypes of macrophages as classically or alternatively activated based on their receptor composition, secretion profiles, morphology and response to external cues. Classically activated inflammatory macrophages have been shown to arise from interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and lipopolysaccharide (LPS) stimulation leading to secretion of pro-inflammatory cytokines and chemokines, often with accompanying tissue damage. Alternative activation of macrophages is stimulated by interleukin-4 (IL-4), IL-10, transforming growth factor-β (TGF-β), or IL-13 and leads to the secretion of anti-inflammatory cytokines, chemokines, growth factors and other reparative factors[26]. In addition to these well characterized stimuli, macrophages in various tissues can be regulated by a wide array of external cues causing phenotypes that may intertwine these subgroups through mechanisms that are not fully understood.

A potentially important but poorly explored set of cues may be neurotrophic factors. Although it has been over two decades since the first studies identified neurotrophin interactions within the immune system,[177] our knowledge of their functions is limited.

Multiple studies have documented expression of various neurotrophins and their receptors in macrophages suggesting that they may play a role in control of the innate immune system [169,172,178-184]. Relatively few studies have looked closely at the functions of these receptors on macrophages. Most information regarding the functions of the neurotrophin receptors comes from studies in the nervous system where neurotrophins are important factors for development, maintenance, survival and differentiation of neurons[121]. The neurotrophin family includes nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). Neurotrophins bind to tyrosine protein kinases known as tropomyosin related kinase (Trk) receptors, TrkA (NGF), TrkB (BDNF and NT-4), and TrkC (NT-3) with high affinity. An additional member of the neurotrophin receptor family, the p75 neurotrophin receptor ($p75^{NTR}$) is a member of the tumor necrosis receptor family and binds all neurotrophins with low affinity. The neurotrophin receptors function as homomeric or heteromeric complexes, providing opportunities for various signaling actions. The $p75^{NTR}$ in particular can interact with any of the Trk receptors where it facilitates receptor activation by increasing the affinity of mature neurotrophin binding.

The neurotrophins are synthesized as precursors (pro-neurotrophins) that must be processed by proteolysis to form the mature protein. All pro-neurotrophins (proNGF, proBDNF, proNT3, proNT4) bind the $p75^{NTR}$ when it associates with alternative co-receptors such as sortilin. Signaling of mature and pro-neurotrophins through their respective receptors often have opposing effects in target cells. Mature neurotrophin signaling has been associated with neuronal survival, growth and differentiation while proNGF signaling often leads to neuronal degeneration and apoptosis [185,186]. These differences have led to the

hypothesis that the balance of pro-neurotrophins versus mature neurotrophins may regulate the course of neurodegenerative diseases [185]. Regulation of macrophage and microglial functions by neurotrophins may be particularly important in the nervous system where neurotrophin expression is high. In addition to neurons, macrophages also secrete neurotrophins. Neurotrophin mRNA expression has been documented in microglial/macrophages in multiple sclerosis plaques [169] as well as HIV-infected macrophages [187]. NGF has been shown to increase CXCR4 mediated migration of macrophage precursor cells, monocytes [183] and to induce the secretion of plasminogen and urokinase-type plasminogen activator from microglia [178]. No studies have yet compared the functional activation of these receptors by pro- versus mature neurotrophins. The following studies were designed to further characterize the expression of neurotrophin receptors on human monocytes and monocyte-derived macrophages (hMDM) and determine the functional role of mature versus pro-neurotrophins. We show that monocytes and macrophages express both $p75^{NTR}$ and TrkA within the same membrane domains and exhibit very different phenotypes in response to mature NGF and proNGF.

Materials and Methods

Isolation and culture of human monocyte-derived macrophages

Human buffy coat leukocytes were purchased and shipped within 24 hours after blood draw from healthy donors at the New York Blood Center (http://nybloodcenter.org/), a non-profit organization for the collection and distribution of blood for clinical and research purposes. All research use was screened by the center and no personal identifiers were sent with the shipment. Blood was diluted 1:1 with phosphate buffered saline (PBS) and was

layered on top of Ficoll-Paque (GE Healthcare 17-1440-03). Blood/Ficoll-plaque was centrifuged at 500 X g for 25 min and the peripheral blood mononuclear cells (PBMCs) were collected from the PBS/Ficoll-Paque interface. PBMCs were washed in red blood cell lysis buffer (Sigma R7757) to remove any red blood cell contamination. PBMCs were centrifuged at 450 X g, the supernatant aspirated and the pellet re-suspended in Dulbecco's modified eagle medium (DMEM) with high glucose, 10% fetal bovine serum (Gibco 160000-044) and 20 μg/ml gentamicin (Gibco 15750-60). Cells were aliquoted into low adhesion 6 well plates (Corning 3471) at a density of approximately 10^7 cells/well. PBMCs were cultured for 5-7 days to allow monocyte attachment. Remaining white blood cells were washed from the plate yielding a pure monocyte/macrophage culture. The adherent cells were differentiated into monocyte-derived macrophages (hMDM) using human GM-CSF (15 ng/ml) in complete DMEM for one week. Monocyte experiments were carried out within 1 hour of PBMC isolation to prevent cell attachment.

Primary cultures of rat forebrain

All animal work was done in accordance with NIH animal welfare guidelines and was approved by the University of North Carolina- Chapel Hill Institutional Animal Care and Use Committee (approval number 14-147.0). Timed gestational embryonic day 9 (E9) pregnant female Long-Evans rats were delivered from Charles Rivers and allowed to rest in UNC animal husbandry until the time of experiments. At gestational day E17, rats were sacrificed by anesthetizing with isoflurane until breathing and heart stopped. The uterus was removed, rinsed briefly in 70% ethanol and placed in HEPES-buffered Hank's balanced salt solution (HBSS) on ice. The brain was removed from each fetus, extensively washed, and the cortex/hippocampus was dissected from each brain and cleaned of Dura-arachnoid membrane

and visible vessels. The tissue was transferred to a 15 ml tube containing 5 ml calcium-magnesium free-HBSS + 2.4 U/ml dispase + 2 U/ml DNase I and incubated for 25-30 min at 36° C. Tissue was triturated and allowed to settle for 2 min. The suspended cells were transferred to a 50 ml culture tube containing 25 ml of minimum essential medium (MEM) with glutamine + 10% fetal bovine serum + 20 µg/ml gentamicin. After several rounds of trituration in 2-3 ml fresh calcium-magnesium free HBSS, dissociated cells were seeded at a density 20,000 cells/cm^2 on poly-D-lysine-treated coverslips for imaging and staining or 50,000-100,000 cells/cm^2 in 100 mm plastic dishes for Western blots. After 24 hours, cultures were transferred to Neurobasal medium with B27 supplement. The resulting cultures were >95% neurons at day 4 after seeding.

Immunostaining

Differentiated hMDM grown on poly-D-lysine coated coverslips were transferred to DMEM containing 1% FBS and stimulated for 1 or 24 hours using three different conditions: NGF human recombinant protein (100 ng/mL, Sigma N1408), proNGF human recombinant protein targeted to high affinity sites (1 ng/ml, Alamone N-280), or vehicle. The cells were gently washed and fixed in 2% paraformaldehyde in PBS. Cells were washed 3X in PBS and incubated in 3% normal goat serum for one hour. Incubation of primary antibodies was carried out overnight at 4° C. Cells were stained using antibodies to: $p75^{NTR}$ (Millipore cat #07-476, 1:500), TrkA (Santa Cruz cat #SC-80961, 1:500), TrkB (Millipore 07-225, 1:500), TrkC (Santa Cruz SC14025, 1:500) and sortilin (Millipore AB9712, 1:500). Cells were washed in PBS and incubated with species specific secondary antibodies conjugated to Alexa 488, 568 or 593 (Molecular Probes) in the dark for 1 hour at room temperature. Coverslips

were then mounted using Fluoromount (Southern Biotech 0100-01) and digitally imaged on an Olympus XI71 microscope.

Analysis of stain intensity and morphology for individual cells or regions of interest within the cells was accomplished using Metamorph software. Co-localization of $p75^{NTR}$ foci with TrkA or sortilin staining was analyzed by staining one receptor red and the other green followed by thresholding by intensity of stain, computer identification of stained objects and then documentation of each object's central X/Y coordinate for each wavelength. Objects with X/Y coordinates that overlapped within 0.46 microns were scored as co-localized.

F-actin and live-dead stains

F-actin was stained using Alexa488 phalloidin (1:50, Molecular Probes) to show structural changes. Podosomes were visible in the hMDM as small, intensely fluorescent puncta whereas ruffles appeared as moderate to brightly stained extensions of the membrane. Cell viability was assessed by incubating cells with the live cell stain, calcein AM (1 μM, Invitrogen/Molecular Probes) and the dead cell nuclear stain, ethidium homodimer (1 μM, Invitrogen/Molecular Probes), for 30 min at 36°. Cells were washed with aCSF and imaged live. Healthy macrophages with bright green fluorescence were counted. The unstained "ghosts" of dead cells were often visible and were counted separately. The number of ethidium stained nuclei was counted to quantify dead cells with multinucleated cells counted as a single cell. Some ethidium stained cells were observed floating on the surface which were difficult to quantify. Thus, the count of live cells was preferentially used as the best

index of cell survival. Some cultures were fixed with 2% paraformaldehyde and stained with 100 nM bisbenzimide to assess total and condensed, fragmented, apoptotic nuclei.

Western blots

Human monocyte-derived macrophages were stimulated with NGF or proNGF for time periods of 0, 10, 30, 60 min and 1 day. At the appropriate time, cells from two 60 mm or one 100 mm dish were harvested using 1.5 ml lysis buffer (1X Ripa buffer (Thermo Scientific 89900), 1 mM phenylmethylsulfonyl fluoride (PMSF, Pierce Chemicals PI 36978), 1:100 Halt® protease inhibitor cocktail (Thermo scientific cat# 1861228). Protein concentration was measured by BCA assay (Thermo Scientific 23225). Protein lysate was resolved on SDS-Page gels (Biorad cat# 456-1034) and transferred to a nitrocellulose membrane (Biorad 162-0112). The membranes were blocked in 2% Bovine Serum Albumin (BSA, Sigma BP1605-100), Odyssey Blocking Buffer (1:1 dilution, Licor 927-40000 for Odyssey) and PBS or milk plus 0.01%Tween (for film) for one hour at room temperature. The primary antibodies were incubated overnight at four degrees. The membranes were then washed in PBS+1% Tween and incubated in secondary antibody for one hour at room temperature. The membranes were washed again and imaged using the Odyssey or film imaging system. The primary antibodies used were 1:500 $p75^{NTR}$ (Millipore 07-476), 1:500 TrkA (Santa Cruz SC80961), 1:1000 Akt (Cell Signaling 9272S), 1:1000 pAkt (Millipore 05-1003). Secondary Antibodies were goat anti-rabbit 680RD (Licor 926-68071) and donkey anti-mouse 800CW (Licor 926-32212), for Odyssey. Film was processed using hoseradish peroxidase (HRP) conjugated secondary antibody and SuperSignal West Pico detection (Thermo Scientific, #34080). .

Phagocytosis of fluorescent beads

Human MDMs were stimulated overnight in 1% DMEM with NGF or proNGF. Fluorescent 1 micron beads (Molecular Probes/Invitrogen F-8887) were placed into each well for 4 hours at a concentration of 4.3 x 10^5 beads/ml. Excess beads not phagocytosed by hMDMs were washed from the plate. The hMDMs were stained with the live cell stain calcein AM (1 μM, 20 min) and digital images of the live cells were captured at a magnification of 674X. Some cells were then fixed in 2% paraformaldehyde and stained with phalloidin-Alexa488 (1:50). Cells were individually traced and Metamorph software was used to measure the number of beads in each cell for each condition. The bead density was calculated by dividing the number of beads by the area of the cell. The average bead density was then calculated and compared between the treatment conditions.

RT-PCR

The hMDM were stimulated in DMEM with 1% FBS plus NGF or proNGF overnight. Cells were lysed using Trizol Reagent (Invitrogen 15596-026) and RNA was purified according to RNeasy protocol (Qiagen cat# 74104). Reverse transcription was carried out according to SuperScript III First-strand Synthesis Kit (Invitrogen cat# 18080-051). The reaction was performed using an ABI 7500 system (Applied Biosystems) and Absolute SYBR Green Rox mix (AB-1163/A). mRNA levels were determined by the cycle threshold. The results were normalized to GADPH. Primers used were TrkA (Forward: 5'ATG CTG CGA GGC CAG CGG CA 3' Reverse:5' CCT GAC AGG GTC AAG TCC TG 3'), TrkB (Forward: 5' CTG GAC CAC GCC AAC TGA CAT 3' Reverse: 5' GCA TCG

GGC CCG CCC TCC GAA 3'), $p75^{NTR}$ (Forward: 5' ATC TTG GCT GCT GTG GTT G 3' Reverse: 5' TGT AGA GGT TGC CAT CAC CC 3'), GADPH (Forward: 5' CCC ATC ACC ATC TTC CAG GA 3' Reverse: 5' TTG TCA TAC CAG GAA ATG AGC 3').

Transmigration Assay

Following an overnight stimulation of hMDMs with NGF or proNGF in DMEM with 1% FBS, cells were harvested by incubating in ice-cold calcium-, magnesium-free HBSS for approximately 20-30 minutes to facilitate release of the hMDM from the low adhesion plate. Cells were washed from the plate with a 1 ml Rainin pipette using the flow of medium from the tip of the pipette to dislodge any remaining cells. The hMDM were centrifuged at 80 x g for 5 min, the supernatant carefully aspirated and the soft pellet was re-suspended in DMEM containing 1% FBS. The cells were counted and seeded into 8.0 micron Matrigel invasion chambers (BD Biocoat cat# 354480) at a density of10^5 cells/chamber. Migratory behavior was measured by the number of cells entering the bottom chamber which also contained DMEM with 1% FBS. Cells in the bottom chamber were labeled with the fluorescent live cell marker calcein AM (Life Technologies, C3100MP, 1 μM 20 minutes) and counted at 1 and 3 days after seeding.

Flow Cytometry

PBMCs and hMDMs were stimulated with NGF or proNGF in 1% DMEM for 1 hour (PBMCs) or overnight (hMDM). Cells were removed from low adhesion wells and centrifuged for 5 minutes at 450 x g. Cellular pellets were re-suspended and fixed in a Fluorfix solution (Biolegend 420801) for 20 minutes at room temperature. Fixed cells were

then treated with permeabilization buffer (EBioscience 020-8333-56) and centrifuged for five minutes at 450 x g at 4° C. The wash step was repeated followed by re-suspension in 100 µl of permeabilization buffer plus antibody (1.5 µl p75; Alomone Labs ANT-007-F and 20µl; TrkA R&D Systems FAB1715P; 5µl CD 206 Biolegend 321114; 5µl CD16 Biolegend 302008 and 5µl CD 163 Biolegend 333607) at room temperature for twenty minutes. The stained cells were washed three times in cell staining buffer (Biolegend 420201). Flow cytometry was performed on a FACS Calibur (Becton Dickinson, San Jose, CA) using direct immunofluorescence with at least 100,000 events. Monocytes were determined by populations of cells with high forward scatter and low side scatter. All cells were gated to remove debris. Three color staining analysis was utilized. Cells were analyzed according to side scatter and receptor bound fluorescence, and data was collected with logarithmic amplifiers. Fluorescence spillover compensation was estimated using single-stained and unstained samples with the Cell Quest software (BD). After collection, data was further analyzed with FlowJo software (TreeStar Inc., Ashland, OR).

Protein profiles of macrophage conditioned medium

The hMDMs were chronically stimulated for 3 days in 1% DMEM with NGF or proNGF. Medium was collected and centrifuged at 400 X g for 5 minutes to remove any floating cells in the medium. The cell free medium was added to a RayBiotech human antibody array L-507 and processed according to the RayBiotech Biotin Label-based human antibody array protocol. Slide arrays were scanned using an Agilent technologies DNA microarray scanner and the analysis was carried out using MetaMorph® software. Internal negative controls were used to establish basal fluorescence and variation across the array.

The minimum detectable fluorescence signal was set at 3.2 standard deviation units above the average background to give a probability of 0.001 that a protein signal would be identified as positive by chance. The linearity of signal detection was verified from internal positive standards. Since signal intensity varied between different arrays, protein expression was normalized to the total signal for all proteins on the array and expressed as a relative optical density value to indicate the strength of each signal. Expression of each protein was compared for NGF or proNGF versus matched untreated controls. A comparison of NGF versus proNGF was then run to determine if the pro and mature peptides activate different secretory pathways. Proteins on the array that met the cutoff for a significant change were clustered into functional groups using DAVID software [188,189] and the relative enrichment of various functional classes determined taking into account the focused nature of the array. Based in part on this information, specific functional subgroups were analyzed in greater detail to determine which classes of proteins were preferentially modified by NGF or proNGF.

Neurotoxicity of macrophage-conditioned medium

Pilot studies indicated that a 30 minute stimulation of hMDM was sufficient to induce secretion of toxic factors which persisted for several hours after removal of the stimulus. Thus, the cells could be primed with neurotrophin, washed and the neurotrophin free medium subsequently tested for activity. Macrophages were stimulated with NGF or proNGF in serum free DMEM for 30 minutes. The medium was then replaced with serum free DMEM and the medium collected after 1 hour or 24 hours. The macrophage-conditioned medium (MCM) was centrifuged at 2500 rpm for 10 minutes to remove any cells and then frozen in

aliquots at -80^{o} C. The neurotoxic activity of the medium was tested on primary rat neurons cultured on coverslips. Neurons at 6-12 days in vitro were loaded with the calcium indicator, Fluo-4 AM (2 μM, Molecular Probes, Inc., Eugene, OR) in aCSF (aCSF: NaCl 137 mM, KCl 5.0 mM, $CaCl_2$ 2.3 mM, $MgCl_2$ 1.3 mM, glucose 20 mM). After 30 minutes, the coverslip was transferred to a specialized stage for imaging. Cells were maintained in aCSF and time lapse digital images were captured automatically by the MetaMorph® System. Images were captured every 6 seconds for 6 minutes to assess acute effects and every min for 60 minutes to assess delayed effects. Three pre-stimulation measurements were taken to establish basal levels of fluorescence at the beginning of each experiment. Neurons were stimulated with MCM at a 1:5 dilution. The increase in fluorescence intensity within each cell was then measured relative to the baseline fluorescence to correct for cell to cell differences in dye loading and intrinsic fluorescence. For most studies, cellular responses were averaged across all cells from at least triplicate runs to provide an indication of the "typical" response. In some cases individual cell response patterns are shown where the average masked important cell-specific profiles.

Macrophage calcium responses to NGF and proNGF

Macrophages cultured on coverslips were incubated with NGF or proNGF and the calcium indicator, Fluo-4 AM in aCSF for 30 minutes. Time lapse digital images were captured automatically and changes in fluorescence intensity within each cell measured as described above.

Statistical analyses

Graphpad Prism software was used for data summaries and graphics. Parametric statistics were used to evaluate most changes induced by NGF or proNGF relative to matched or unmatched control samples depending on the experiment. In cases where the data were not normally distributed non-parametric statistics were used. Means $\pm$ standard error of the mean were calculated for at least three replicate experiments. T-tests were used for paired comparisons, analysis of variance with repeated measures for temporal data and Chi-square for the analysis of cell populations as indicated. Unless otherwise indicated, a probability of <0.05 for rejection of the null hypothesis was considered significant.

Results

Macrophages display distinctly different morphological phenotypes in culture

In culture, hMDM exhibited different morphologies which were, in part, dependent on the culture and stimulation conditions. Examples of prevalent morphological features are summarized in Figure 1.1. Three major structural features were identified which were found to correlate with macrophage phenotype: 1) ruffled membranes on the surface and edges of the cell and cellular processes (Fig 1.1A, arrows), 2) "fried egg" morphology with finger-like projections along the outer edges of the membrane (Fig 1.1B, arrow) and 3) flat amoeboid cells with few readily visible membrane specializations (Fig 1.1C). The full range of morphologies could often be seen in the same culture although under particular stimulus conditions a single morphological type typically predominated. Occasionally, individual cells would display a combination of the above features although a single feature would usually dominate. To provide insight into the potential functional role of these specializations, each morphological feature was related to particular macrophage phenotypes described below.

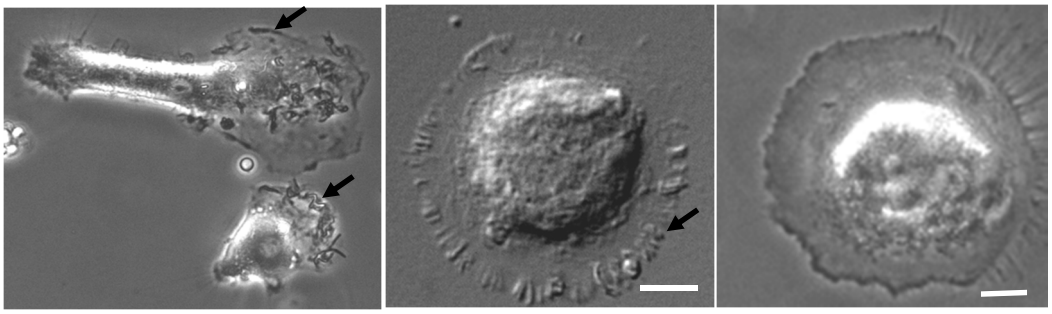


Figure 1.1. Varied appearance of human monocyte-derived macrophages (hMDM) in culture. A-C. Bright field images of hMDMs in culture illustrating ruffled membranes (A, arrows), flat, fried egg-like morphology with finger like folds in the outer membrane (B, arrow) and flat amoeboid appearance with few membrane specializations (C). Scale bar = 20 μm in all images.

TrkA and $p75^{NTR}$ are co-localized to discrete domains on macrophages

To investigate the expression and localization of neurotrophin receptors, we stained hMDMs for each class of neurotrophin receptor. Strong focal expression of $p75^{NTR}$ (Fig 1.2A) and TrkA (Fig 1.2B) was seen in regions of the macrophages containing ruffles (Fig 1.2C, arrow) or finger-like membrane specializations (see Fig 1.6). Although both receptors were expressed within the same structural domain, $p75^{NTR}$ expression was typically more focal and surrounded by a more diffuse TrkA stain (Fig 1.2D). TrkA and $p75^{NTR}$ staining were also seen in small endosome-like structures throughout the cell (Fig 1.2D, arrowhead). TrkB immunoreactivity was low with little or no expression localized to ruffles (Fig 1.2E, arrow) and occasional staining in endosomal-like structures (Fig 1.2E, arrowhead). Staining for TrkC was consistently negative as were control hMDM stained with secondary antibody in the absence of primary antibody (Fig 1.2F). It is notable that the structural specializations were highly dynamic. Even washing the cells too aggressively could trigger changes in shape and morphology as well as the ability to detect foci of both $p75^{NTR}$ and TrkA. It was difficult to unambiguously identify staining in large flat cells that had no clear membrane specializations (Fig 1.2G, outline of cell marked with dashed lines). Overall, the percentage of macrophages with readily identifiable staining was 47.4% $p75^{NTR}$-positive and 44.3% TrkA-positive. Macrophage expression of $p75^{NTR}$ and TrkA was confirmed by Western blot (Fig 1.2H). RT-PCR also verified mRNA transcript for $p75^{NTR}$ and TrkA with comparable delta cycle thresholds for each receptor (11.54 ± 0.54 and 11.95 ± 0.84, respectively). Consistent with the staining results, weak or no significant mRNA expression was detected for TrkB or TrkC.

Since $p75^{NTR}$ and TrkA are potential signaling partners, the extent of co-localization of $p75^{NTR}$ and TrkA was determined in double-stained macrophages. At the whole cell level, 56.3 $\pm$ 8.0% of the hMDM expressed both TrkA and $p75^{NTR}$. However, to determine if TrkA and $p75^{NTR}$ were co-localized to the same microdomains, the overlap of the stained foci was determined with a resolution of $\pm$ 0.46 μm using MetaMorph® software. TrkA was co-localized to 15.7 $\pm$ 4.8% of the $p75^{NTR}$ foci (range 4.9 to 33.6%). Some of the co-localization of $p75^{NTR}$ and TrkA was seen in small endosome-like structures. Thus, while $p75^{NTR}$ and TrkA were expressed in the same general location in most cells (e.g. Fig 1.2C) only a subset of the receptors overlapped.

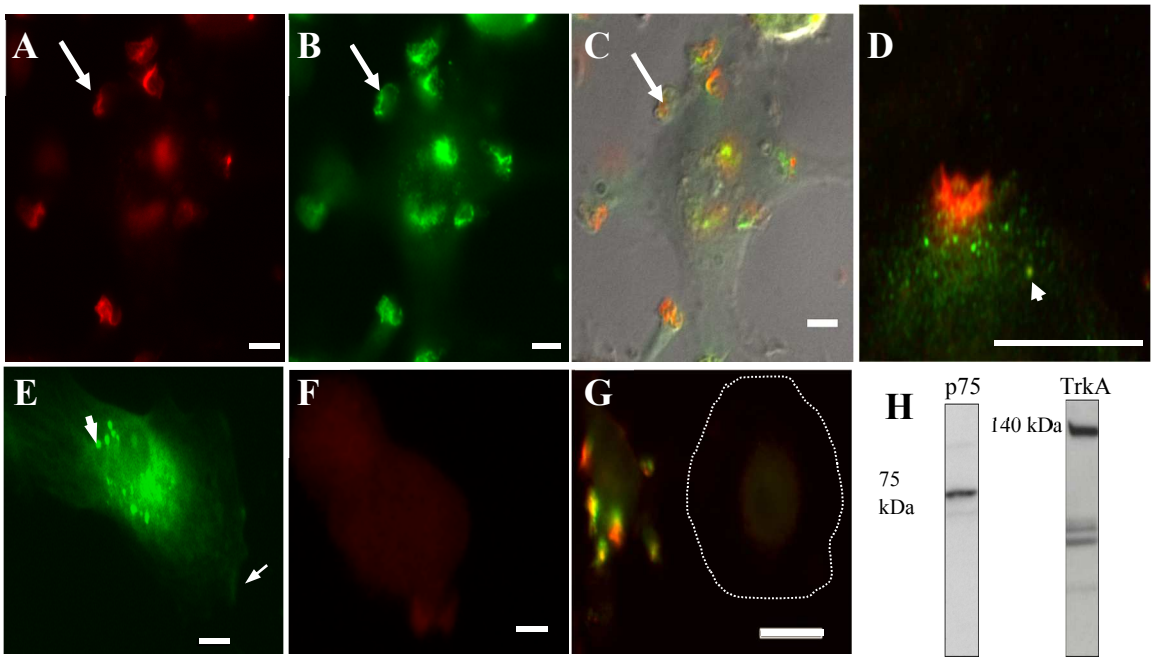


Figure 1.2. Neurotrophin receptors $p75^{NTR}$ and TrkA were concentrated in discrete domains on human macrophages. A-C. A single macrophage stained for $p75^{NTR}$ (A, red), TrkA (B, green) illustrating the localization of immunoreactivity to ruffled areas . C. DIC image overlay showing the merged receptor stain for $p75^{NTR}$ (red) and TrkA (green) associated with membrane ruffles on the surface of the macrophage. D. Highly magnified view of the outer membrane of a macrophage showing $p75^{NTR}$ (red) immunoreactivity surrounded by more diffuse labeling of TrkA (green). Immunoreactivity for $p75^{NTR}$ and/or TrkA (arrowhead) was seen in endosome like structures at these foci as well as throughout the cell. E. Immunoreactivity for TrkB was typically weak with little expression at membrane specializations (arrow). However, a few cells showed immunoreactivity in endosome-like structures within the cell (arrowhead). F. hMDMs stained with secondary antibody in the absence of specific primary antibody showed no significant immunoreactivity. G. hMDM with no expression of $p75^{NTR}$ or TrkA within the cell or along the outer membrane (membrane border identified with dotted lines). The cell is adjacent to a stained cell illustrating the variable nature of the expression of $p75^{NTR}$ and TrkA. H. Western blots of hMDM lysate confirming immunoreactive bands at 75 kDa and 140 kDa for $p75^{NTR}$ and TrkA, respectively.

p75^{NTR} and TrkA are expressed on monocytes

Expression of p75^{NTR} and TrkA on hMDM was confirmed with flow cytometry using a different set of antibodies. By flow cytometry, 53.0 $\pm$ 8.5% of the macrophages were p75^{+} and 14.1 $\pm$ 1.7% was TrkA^{+}. To explore whether p75^{NTR} and TrkA were also present on monocytes, the precursors of mature macrophages, we used flow cytometry to measure the expression on freshly isolated PBMCs from healthy donors. Cells were stained and analyzed from seven separate donors with similar results. On average, 59.2 $\pm$ 14.9% of the gated monocyte subset was p75^{+} and 29.5 $\pm$ 12.6% was TrkA^{+}. The p75^{NTR} expression was similar to the expression seen on the macrophages whereas TrkA expression was higher on the monocytes. The cellular co-localization of p75^{NTR} and TrkA by flow indicated that, on average, 88.8% of the TrkA^{+} monocytes were also positive for p75^{NTR}. This was slightly higher than the 56.3% cellular co-expression seen in the immunostained hMDM.

The above results established the expression of p75^{NTR} and TrkA under normal culture conditions. The presence of both p75^{NTR} and TrkA suggested that macrophages may be subject to the differential actions of NGF and proNGF as suggested for neurons. Therefore, we assessed responses to stimulation with NGF or proNGF to determine how these neurotrophins influence the phenotype and functions of the macrophages.

Survival versus death in human monocyte derived macrophages

To determine if neurotrophins support survival of macrophages as they do for neurons, hMDM were cultured in serum free medium in the presence or absence of various concentrations of NGF (0, 3, 10, 30 or 100 ng/ml). After 28-32 hours, hMDM were stained with the live cell marker, calcein AM, imaged, and normalized to control hMDM maintained

in medium containing 10% FBS. The percentage of live, healthy hMDMs stimulated with NGF in culture medium lacking serum increased in a dose dependent manner from 38.1 ± 10.7% at 0 ng/ml NGF to 101.2 ± 6.0% at 100 ng/ml NGF (Fig 1.3A) with an estimated EC_{50} of 14.0 ng/ml. A separate set of hMDM maintained in medium containing 10% FBS and stimulated with each concentration of NGF showed no changes at any concentration with a mean survival of 100.6 ± 2.2% (relative to control hMDMs). In parallel with the survival data, a dose-dependent decrease in ethidium homodimer staining was seen with maximal protection at 100 ng/ml NGF (not shown).

The pro-survival actions of NGF were consistent with the phosphorylation of Akt, a known pathway for NGF mediated survival in neurons. However, as illustrated in Fig 1.3B, stimulation with NGF or proNGF both resulted in a time-dependent increase in phosphorylation of Akt (33.2 ± 7.0% and 69.0 ± 31.2%, maximal increase respectively, p=0.032) relative to controls, followed by recovery (Fig 1.3B). This suggested that neurotrophin receptors $p75^{NTR}$ and TrkA both mediate pro-survival functions on hMDMs.

Since signaling of pro-neurotrophins through the $p75^{NTR}$ receptor can promote cell death in neurons, we stained hMDMs stimulated with neurotrophins overnight with the dead cell marker ethidium homodimer and then fixed the cells in 2% paraformaldehyde before staining with the nuclear stain bisbenzimide to identify all nuclei as well as apoptotic nuclei. On average, 10.1 ± 1.4% of the untreated hMDM nuclei stained positive for ethidium homodimer. This value did not change significantly after overnight treatment with 1 ng/ml proNGF (9.6 ± 1.2%) or 100 ng/ml NGF (12.2 ± 2.2%) (Fig 1.3C). Condensed and/or fragmented nuclei represented 7.2 ± 1.1% of the total population in the vehicle-treated cells

and also did not change with proNGF or NGF treatment (6.5 $\pm$ 1.0% and 7.3 $\pm$ 1.2%, respectively; Fig 1.3D).

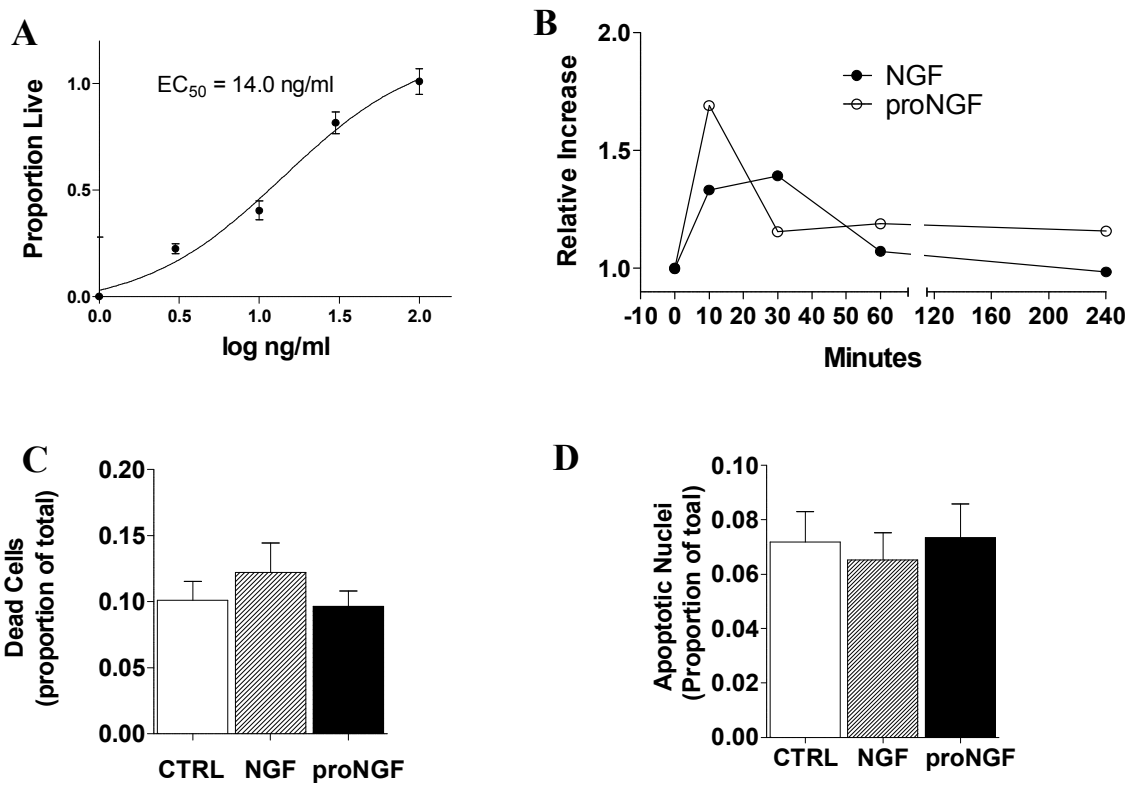


Figure 1.3. Neurotrophins promote cell survival in hMDMs. A. NGF increases survival of hMDMs in a dose response (0ng/ml,3ng/ml,10ng/ml,30ng/ml and 100ng/ml) manner. EC_{50}=14.0ng/ml (n=9). B. Time course (0,10, 30, 60, 240 min) of changes in the ratio of pAKT: total AKT levels after addition of 100ng/ml NGF or 1ng/ml proNGF. Phosphorylation (activation) of AKT determined from quantification of bands in western blots was increased with NGF or proNGF in each of five different runs followed by recovery. NGF stimulation resulted in a longer lasting phosphorylation of AKT. Results are expressed as mean ± sem. p=0.032 treatment effect over time (n=4). C. Live cultures of treated or untreated hMDM were stained with the dead cell marker, ethidium homodimer. The total number of dead cell nuclei stained with ethidium was low and was not changed after 1 day stimulation with 100ng/ml NGF or 1ng/ml proNGF (n=12). D. The same cultures of hMDMs were fixed and counterstained with the nuclear stain bis-benzamide to reveal apoptotic nuclei. The number of condensed, fragmented apoptotic nuclei was very low and did not change after stimulation with 100ng/ml NGF or 1ng/ml proNGF (n=12).

Expression of the $p75^{NTR}$ and TrkA in monocytes and macrophages stimulated with NGF or proNGF

To investigate whether neurotrophin stimulation of human monocytes or macrophages would alter neurotrophin receptor expression, we stimulated hMDM with 100 ng/ml NGF or 1 ng/ml proNGF for 24 hours and analyzed receptor expression by flow cytometry (Figure1. 4A). Untreated cells were 53.0 $\pm$ 8.5% $p75^{+}$ and 14.1 $\pm$ 1.7% $TrkA^{+}$. NGF stimulation of hMDMs resulted in a small but significant decrease in $p75^{NTR}$ expression relative to both controls (-20.4 $\pm$ 7.3%, p=0.046) and proNGF (-22.5%, p=0.053). Expression of TrkA was not significantly affected (-11.2 $\pm$ 16.8%). Treatment with proNGF did not have a significant effect on $p75^{NTR}$ or TrkA expression (+2.6 $\pm$.08% and +0.6 $\pm$13.6%, respectively).

Freshly isolated PBMCs were also stimulated with NGF or proNGF for 1 hour and total gated monocytes were analyzed. Untreated cells were 59.2 $\pm$ 14.9% $p75^{+}$ and 29.5 $\pm$ 12.6% $TrkA^{+}$. NGF and proNGF did not affect $p75^{NTR}$ or TrkA total expression (Figure1. 4B). However, as illustrated in Figure 5, monocytes positive for $p75^{NTR}$ (Fig. 1.5A) or TrkA (Fig. 1.5B) could be subdivided into two subpopulations expressing low and high receptor content. NGF stimulation slightly increased the proportion of $TrkA^{high}$ expressing monocytes (Fig 1.5C) compared to both matched controls (+14.1 $\pm$ 21.6% p=0.021) and proNGF (21.6% p=0.02). ProNGF stimulation did not affect the $p75^{high}$ or $TrkA^{high}$ populations (Fig 1.5C).

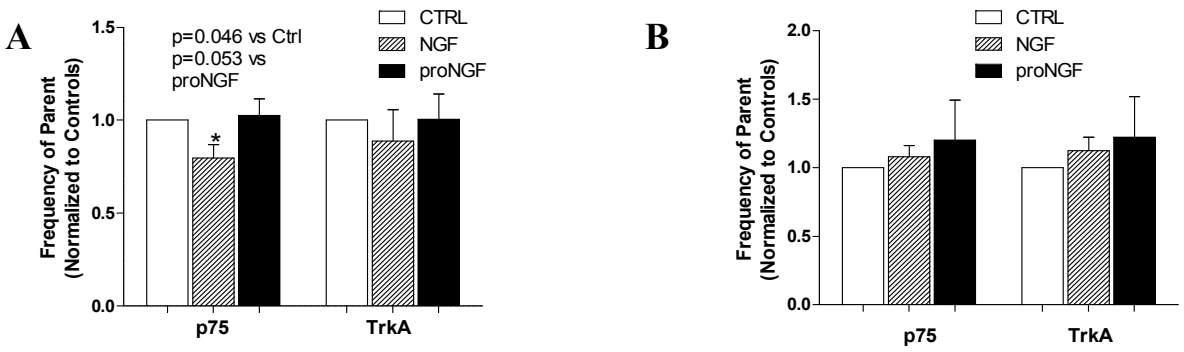


Figure 1.4. Neurotrophin receptor expression on hMDMs and monocytes quantified by flow cytometry. Receptor expression of TrkA and $p75^{NTR}$ was assessed by flow cytometry analysis on cultured hMDMs (A) and freshly prepared human PBMCs (B). A. hMDMs stimulated with 1ng/ml proNGF or 100ng/ml NGF for 1 day had no effect on TrkA expression. NGF stimulation reduced $p75^{NTR}$ expression (n=7). B. Human monocytes stimulated with 100ng/ml NGF or 1ng/ml proNGF for 1 hr showed no significant change in total receptor content of $p75^{NTR}$ or TrkA. (n=6).

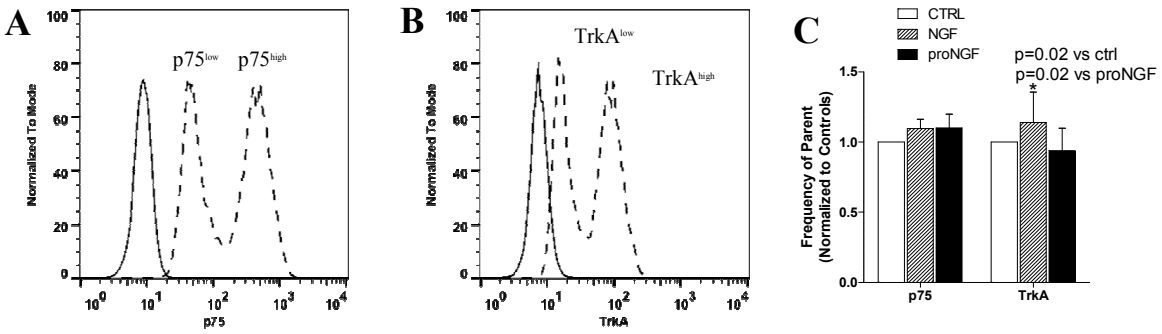


Figure 1.5. Monocytes expressed high and low levels of $p75^{NTR}$ and TrkA. Two populations of positively labeled neurotrophin receptors were seen by flow cytometry. A. Representative histogram of $p75^{NTR}$ low and high populations seen in monocytes relative to unstained cells. B. Representative histogram of TrkA low and high populations (dashed lines) seen in monocytes relative to unstained cells (solid lines). C. Monocytes stimulated with 100ng/ml NGF or 1ng/ml proNGF for 1 hr had no effect on the proportion of $p75^{high}$ expressing monocytes. NGF stimulation induced a small but significant increase in monocytes with high expression of TrkA. (n=7).

Co-localization of the $p75^{NTR}$ and TrkA on hMDM is influenced by NGF and proNGF

At the cellular level by flow cytometry we did not see a significant difference in $p75^{NTR}$/TrkA co-expressing cells; however, we sought to understand whether regional co-localization of $p75^{NTR}$ and TrkA immunoreactivity within individual hMDM was sensitive to neurotrophin stimulation. Cultured hMDMs were double stained for $p75^{NTR}$ and TrkA, and the overlap of the staining (Figure 1.6A) was analyzed at a resolution of 0.46 μm (Merged inset). In the absence of stimulation, an average of 15.7 $\pm$ 4.8% of $p75^{NTR}$ staining co-localized to TrkA foci (Fig 1.6B). When the hMDM were exposed to NGF or proNGF, co-localization of TrkA with $p75^{NTR}$ increased significantly to 60.0 $\pm$ 2.4% and 30.4 $\pm$ 5.1%, respectively, ($p<0.001$ for both). The co-localization of $p75^{NTR}$ and TrkA induced by NGF was twice that of proNGF, ($p<0.001$) indicating a preferred mobilization of these receptors to the same domain by NGF. The double-labeled profiles were typically seen in ruffled regions and small endosome-like structures as described above (Figure 1.2).

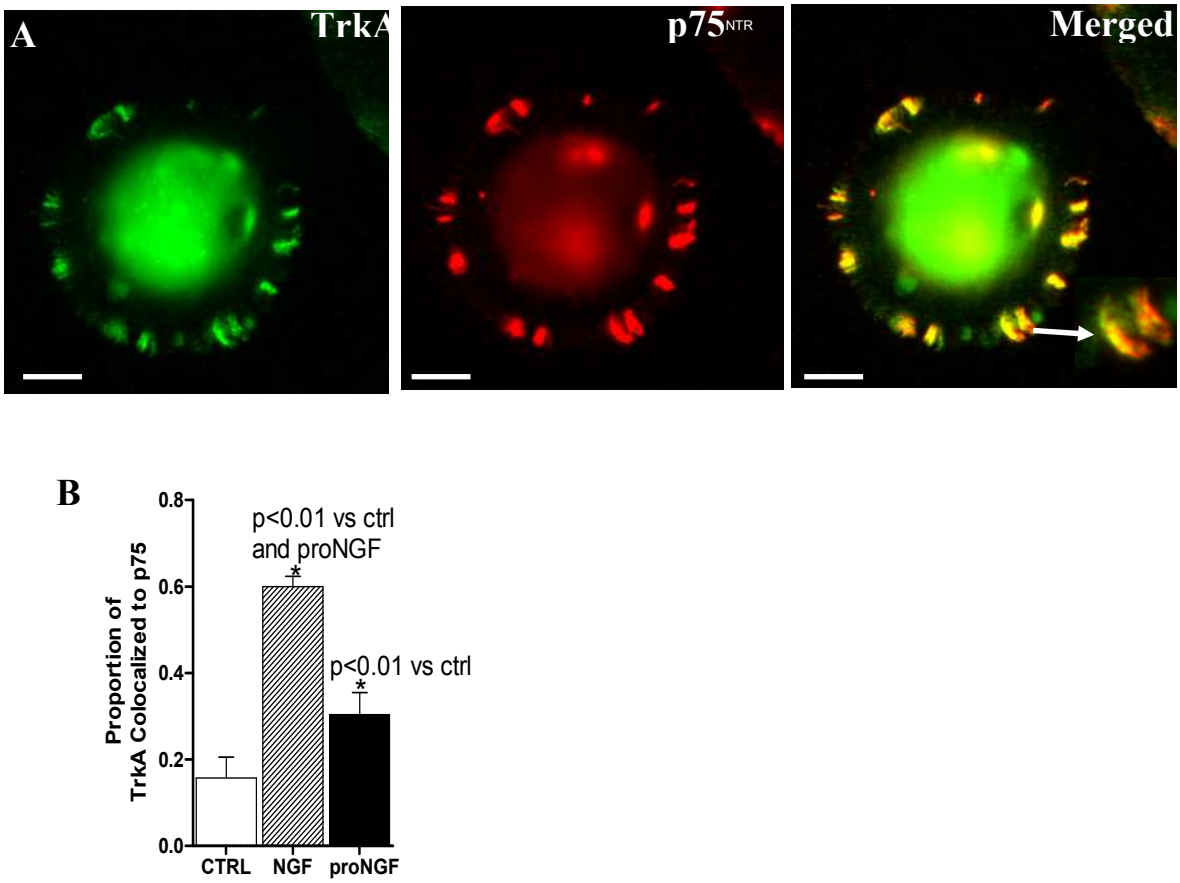


Figure 1.6. Neurotrophin receptors $p75^{NTR}$ and TrkA are co-localized to the same microdomains on hMDMs. A. Immunoreactivity of TrkA (green), $p75^{NTR}$(red) and colocalized foci of $p75^{NTR}$ and TrkA (yellow) found on finger-like membrane specializations of hMDMs. At higher magnification (inset) the partial overlap of $p75^{NTR}$ and TrkA can be seen indicating that the two receptors are closely associated but only occasionally within the same microdomain. B. The ratio of TrkA labeling co-localized with $p75^{NTR}$ at a resolution of 0.46 µm was quantified. 100ng/ml NGF significantly increased co-localization compared to control and 1ng/ml proNGF after 1 day. A smaller yet significant increase in co-localization of TrkA and $p75^{NTR}$ was seen with proNGF(n=12).

Sortilin receptor expression on hMDM

A well-recognized action of proNGF in some cell types is fostered by the interaction between $p75^{NTR}$ and the sortilin receptor. To determine if sortilin receptors were also present on human macrophages, we evaluated its expression by immunostaining. Sortilin was expressed at moderate levels on hMDMs, typically in small focal regions associated with ruffles (Figure 1.7A). A low level of diffuse stain was often seen throughout the cell. High to moderate sortilin expression was seen in approximately 43% of the cells whereas 57% of cells showed weak staining. Since the focal expression of sortilin on ruffles was similar to $p75^{NTR}$ expression, double staining was performed to co-localize the $p75^{NTR}$ and sortilin receptors (Figure 1.7A, Merged and Figure 1.7B). On average 16.3 $\pm$ 1.4% of the $p75^{+}$ foci overlapped with sortilin (arrows). However, the degree of overlap within each cell varied widely from 0 to 100%. Most cells (86%) had less than 20% overlap of the two receptors. Treatment of the hMDM with NGF or proNGF had differential effects on sortilin expression and co-localization with $p75^{NTR}$. NGF increased the co-localization of $p75^{NTR}$ with sortilin by 104% (Fig. 1.7B, $p<0.001$) relative to controls and 42.6% relative to proNGF ($p<0.001$; arrows, Fig 1.7B). ProNGF also significantly increased co-localization relative to controls by 49.8% ($p<0.001$, Fig 1.7B). Treatment with NGF and proNGF had similar effects on co-localization of the receptors at 24 hrs indicating that the effects were relatively rapid and persistent. Total sortilin expression, based on the fluorescence stain intensity, was slightly (-10%) yet significantly reduced by NGF (Fig. 1.7C, $p<0.001$) but increased 36.8% by proNGF relative to controls (Fig 1.7C, $p<0.001$) and 50.8% relative to NGF ($p<0.001$). As noted above, these conditions did not lead to an increase in cell death suggesting that interactions with sortilin may serve other functions.

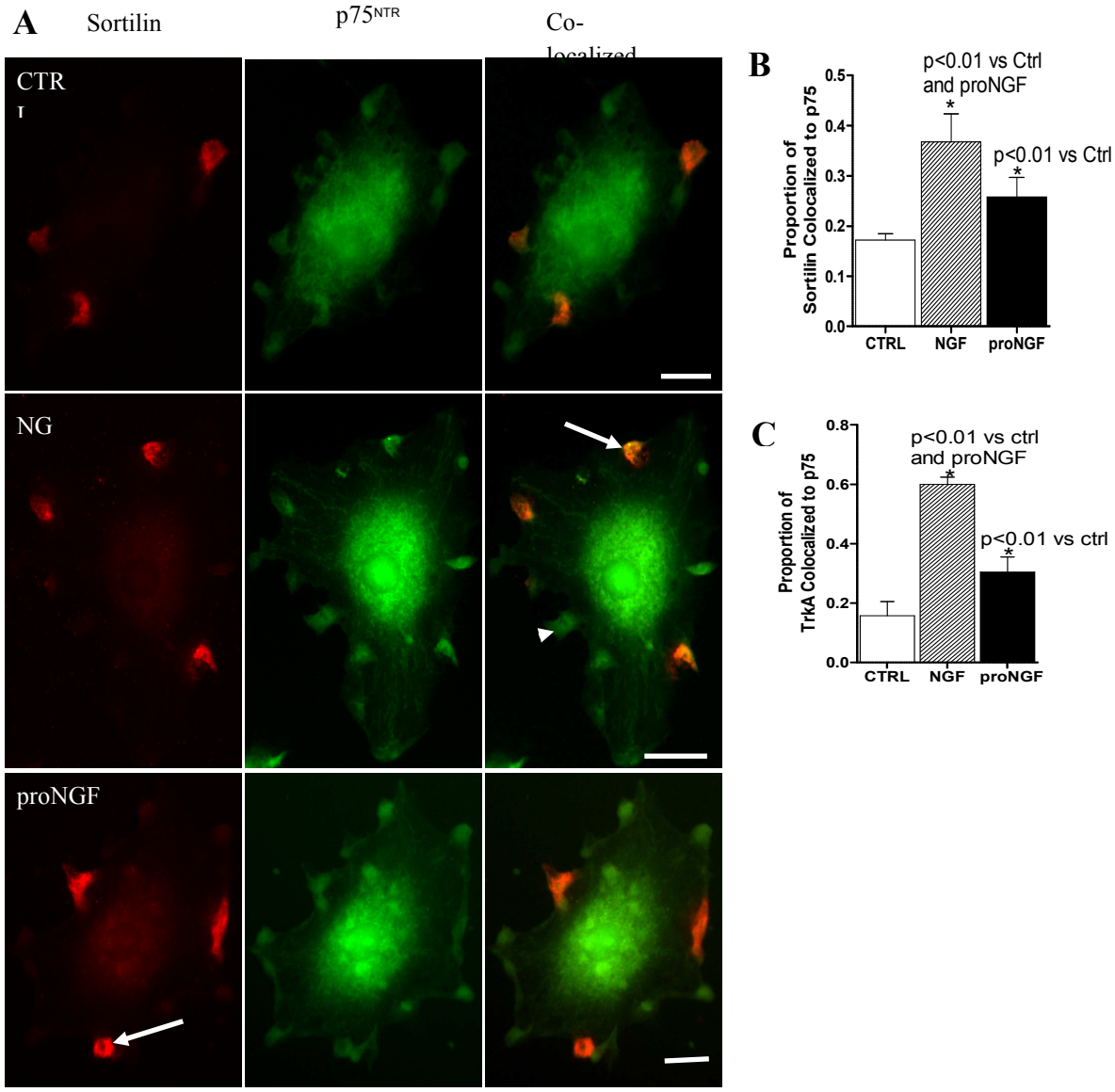


Figure 1.7. Sortilin receptors are expressed and co-localize with $p75^{NTR}$ on hMDMs. A. Example of focal immunostaining for sortilin (Red) and p75 (Green) in control hMDM and hMDM treated with 100ng/ml NGF or 1ng/ml proNGF for 1 h. B. Overlap of p75 and sortilin staining was quantified as in Figure 5. 100ng/ml NGF and 1ng/ml proNGF stimulation increased the ratio of sortilin co-localized with p75 (6A arrows) compared to control. Many p75 foci were not co-localized with sortilin (6A; n=15). C. Sortilin immunoreactivity expression intensity after 1 h stimulation with 100ng/ml NGF or 1ng/ml proNGF was quantified in individual cells. The mean immunoreactive intensity of sortilin was increased by proNGF after 1 hour (6A, ProNGF arrow; n=15). Results are expressed as mean $\pm$ sem.

Neurotrophin modification of macrophage structure

To view changes in macrophage structure as a result of neurotrophin stimulation, cultured hMDM were stimulated with NGF or proNGF for a period of 1 hour or 24 hours followed by fixation in 2% paraformaldehyde and staining for F-actin with phalloidin-Alexa488. Structural changes were seen within the first hour after stimulation. Examples of the appearance of the hMDMs are illustrated in Figure 1.8A. F-actin (green) is shown as well as the presence of red fluorescent beads which were used to measure phagocytosis (Fig 1.9A). Vehicle-treated control cultures initially contained a mixture of cells with podosome-like structures (22.9% of cells, Fig 1.8A, podosome rich), focal ruffles (37.0% of cells, Fig 1.8A, ruffled), or no distinctive membrane specializations (40.1% of cells, Fig 1.8A, no specializations). Cells with podosomes often exhibited a highly polarized expression of podosomes (Fig 1.8A, polarized). In untreated cultures similar numbers of ruffled versus podosome-containing cells were seen with an average ruffled: podosome ratio ranging from 0.94 to 1.61. After 1 hour in the presence of 100 ng/ml NGF, 27.8% of the cells predominantly expressed ruffles and 10.5% podosomes, yielding an increased ruffled to podosome ratio of 2.65 $\pm$ 0.06 (Fig 8B, $p<0.001$, compared to controls). This was largely due to a decrease in the expression of podosomes. Stimulation with NGF for 24 hrs resulted in a similar ruffled to podosome ratio of 2.14 $\pm$ 0.06_(Fig 8B, $p<0.001$ NGF vs. control or proNGF) although in this case there was a large increase in the number of hMDM with ruffles. After exposure to proNGF for 1 hour the opposite pattern was seen with 44.7% of the cells expressing podosomes and 24.2% ruffles decreasing the ruffled to podosome ratio to 0.54 $\pm$ 0.14 (Fig 1.8B, $p<0.001$, vs. Control or NGF). However, 24 hour stimulation with proNGF did not affect the ruffled to podosome ratio relative to controls (Fig 1.8B, 0.83 $\pm$

0.17 vs. 0.94 ± 0.20, respectively). The effect of NGF on ruffle formation was further illustrated by a concentration-dependent increase in the number of cells with ruffles from 36.4 ±4.2% at 0 ng/ml NGF to 79.7 ± 7.3% at 100 ng/ml NGF (Fig 1.8C). The increase in ruffled cells seen after 100 ng/ml NGF stimulation (62.0±0.46 %, Fig 1.8D, p=0.028) was blocked by inhibiting TrkA signaling with a specific inhibitor, GW 441756 (Fig 1.8D, p=0.01 NGF vs NGF+ GW 441756). This data indicated that NGF drives the formation of actin rich ruffled structure via TrkA signaling in hMDMs.

Although NGF and proNGF produced different effects on the expression of podosomes, both increased the polarization of podosome-like structures after 1 and 24 hours. When ranked based on the degree of polarization from 1 (no polarity) to 5 (highly polarized - podosomes concentrated in one quadrant of the cell), controls had little polarization (1.25 ± 0.04). After 24 h, NGF- and proNGF-treated cells showed increased polarization (1.71 ± 0.12, p=0.0248 and 2.27 ± 0.12%, p<0.001, respectively, Fig 1.8E). The degree of polarization was significantly greater for proNGF versus NGF (p<0.001). This trend was particularly evident in the relative number of cells in the 3-5 polarization categories: 3.38%, 20.17% and 35.09% for control, NGF and proNGF, respectively.

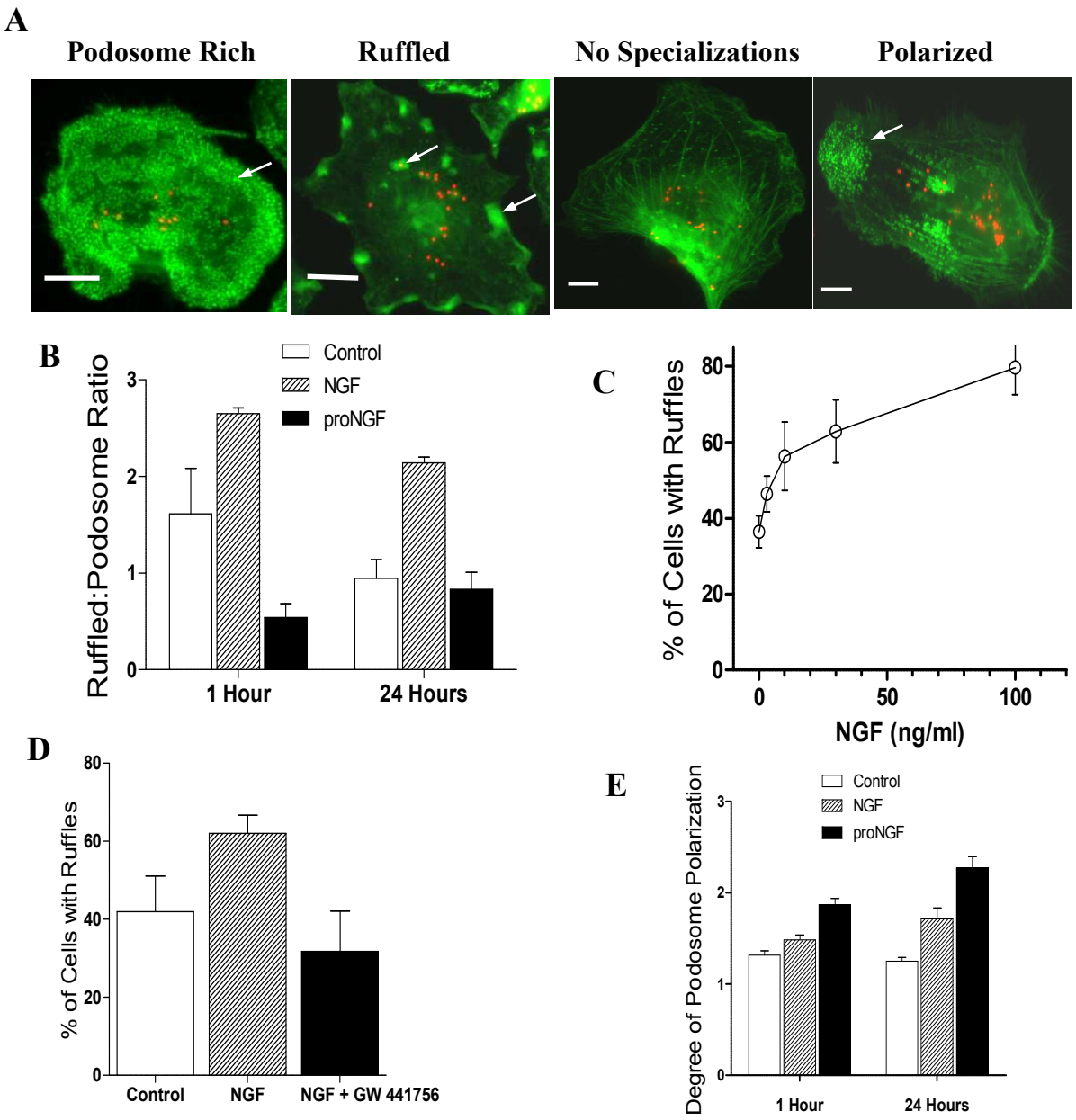


Figure 1.8. Morphological changes in hMDMs are regulated by neurotrophins. A. hMDMs stained with the F-actin marker phalloidin (Green) following phagocytosis of 1micron latex beads (Red). Four morphologies were often seen in cultures: hMDMs rich in podosomes (podosome rich, arrow), hMDMs containing focal ruffles (ruffled, arrows), hMDMs with no significant membrane specializations (no specializations) and polarized hMDM with podosomes concentrated at one end of the cell (polarized, arrow). Small red fluorescent beads are visible reflecting phagocytosis summarized below. B. The expression of ruffles versus podosomes in hMDMs changed in opposite directions in response to 100ng/ml NGF or 1ng/ml proNGF as summarized by the ratio of ruffled cells to cells predominantly expressing podosomes. An increase in ruffled cells by NGF was seen after 1 and 24 h. An

increase in podosome rich cells was seen 1 h after treatment with proNGF but was not sustained. (n=10). C. Dose response increase in the percentage of F-actin rich ruffled containing hMDMs after stimulation with various concentrations of NGF (0ng/ml,3ng/ml,10ng/ml,30ng/ml,100ng/ml) (n=6). D. 100ng/ml NGF increased the number of cells containing ruffles. Specific inhibition of TrkA signaling with GW 441756 in the presence of 100ng/ml NGF decreases the percentage of cell with ruffles compared to NGF alone and controls. (n=4). E. To assess polarization of the hMDMs, cultured cells containing podosomes were scored from 1(no polarity) to 5 (high podosome density localized to one quadrant of the cell).100ng/ml NGF and 1ng/ml proNGF both increased polarization of hMDM after 1 h, and 24 h of stimulation. ProNGF induced a greater polarization compared to NGF. (n=20).

Phagocytosis of fluorescent beads

Macrophages are important in removal of cellular debris and infectious agents through phagocytosis, and the appearance of ruffles has been associated with calcium-dependent phagocytic activity[190]. To verify that the ruffled morphological phenotype was associated with phagocytic activity, we measured the phagocytosis of 1 micron fluorescent beads over a period of 4 hours. Cells were stained with calcein AM for live cell imaging and/or fixed and stained with phalloidin to determine ruffled vs non-ruffled cells (e.g. Fig 1.8A). Cells containing ruffled specializations phagocytosed more beads (Fig 1.9A) than non-ruffled cells (64,295 $\pm$ 2,825 and 31,552 $\pm$ 5,861 beads/mm^2, respectively * $p<0.001$).

MDM transmigration across a matrigel barrier

As podosomes have been implicated in migration and invasion, we investigated the roles of NGF and proNGF during macrophage migration. Macrophages were incubated in DMEM +1% FBS with or without proNGF or NGF. An aliquot of 10^5 cells from each condition was added to the upper well of an 8.0 micron matrigel invasion chamber for 1 or 3 days with similar medium in the lower well. NGF increased migration but did not reach significance relative to untreated controls. ProNGF had no effect on migration after 1 day but significantly increased the transmigration of hMDM relative to controls (104.8% increase, $p=0.051$) and NGF treated cells (149% increase, $p=0.011$) by day 3 (Fig 1.9B). Thus, the transmigration of the cells was consistent with the expression and polarization of podosomes by proNGF.

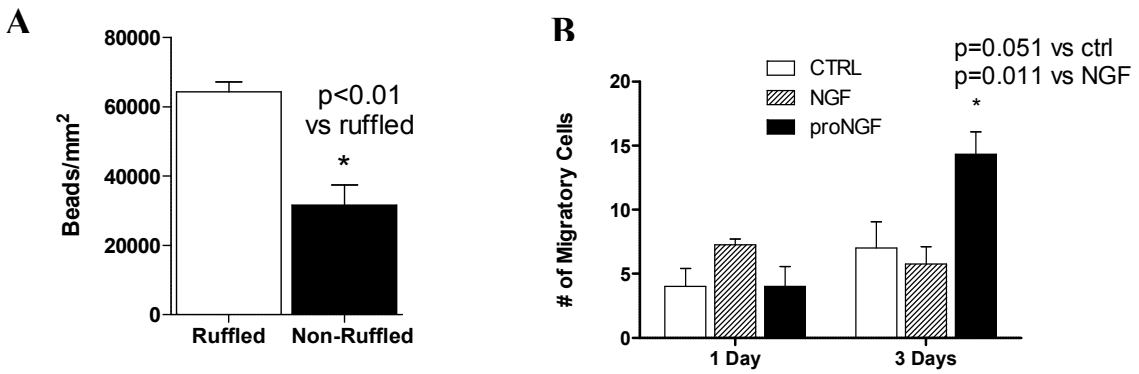


Figure 1.9. Functional changes by NGF and proNGF. A. Phagocytosis of 1 μm fluorescent latex beads (red) by hMDMs with ruffled morphologies was significantly greater than non-ruffled hMDMs B. The number of migratory hMDMs was increased after 3 days stimulation with 1ng/ml proNGF compared to controls or 100ng/ml NGF (n=4).

NGF and proNGF regulate intracellular calcium spiking activity in macrophages

Calcium signaling has been linked to various types of activity in macrophages. Macrophages responded to both NGF and proNGF with increases in intracellular calcium but the patterns were different. A plot of the average calcium responses (61-92 cells/condition), illustrated in Figure 1.10A, showed that both NGF and proNGF provoked a small acute response (Fig 1.10A and B, arrow) that was not seen in vehicle-treated macrophages. Over time, both the untreated and NGF-treated macrophages showed a gradual increase in intracellular calcium that was not apparent in the presence of proNGF (Fig 1.10A). The average acute increase in calcium was 32.5 $\pm$ 3.4 fluorescence units in hMDM stimulated with NGF and 34.3 $\pm$ 8.0 in hMDM stimulated with proNGF (Fig 1.10C, p's < 0.001 relative to control, 1.2 $\pm$ 1.9). A gradual rise in intracellular calcium over time was seen in 39.1% of the control hMDM. Many fewer cells showed a gradual accumulation in the presence of NGF (16.9%) and the gradual rise was almost absent in the presence of proNGF (1.6%)($p<0.001$, Chi square). These average response profiles, however, obscured the presence of brief calcium spikes that occurred at random intervals. Comparison of the spiking patterns in individual cells showed considerable variation in the magnitude and frequency of the calcium responses. Examples of the various patterns seen are provided in Figure 1.10B. To quantify the different response patterns, we examined the acute responses (Fig 1.10C) and spiking frequency (Fig 10D). The average acute calcium increase was significantly greater than controls for both NGF and proNGF (Fig 1.10C). Calcium spikes were seen in 39.1% of the unchallenged hMDM and 39.8% of NGF-treated cells but was reduced to 16.4% in the presence of proNGF ($p=0.0027$, Chi square). A frequency analysis of the individual calcium spike magnitudes indicated that NGF shifted the calcium spikes to

higher magnitudes whereas proNGF shifted the responses to lower magnitudes ($p < 0.001$, Chi square). The spike frequency per cell was also greater for NGF versus proNGF (Fig. 1.10D, $p=0.0149$). Thus, both the number of cells showing calcium changes and the temporal nature of the calcium response were altered in different directions by NGF versus proNGF.

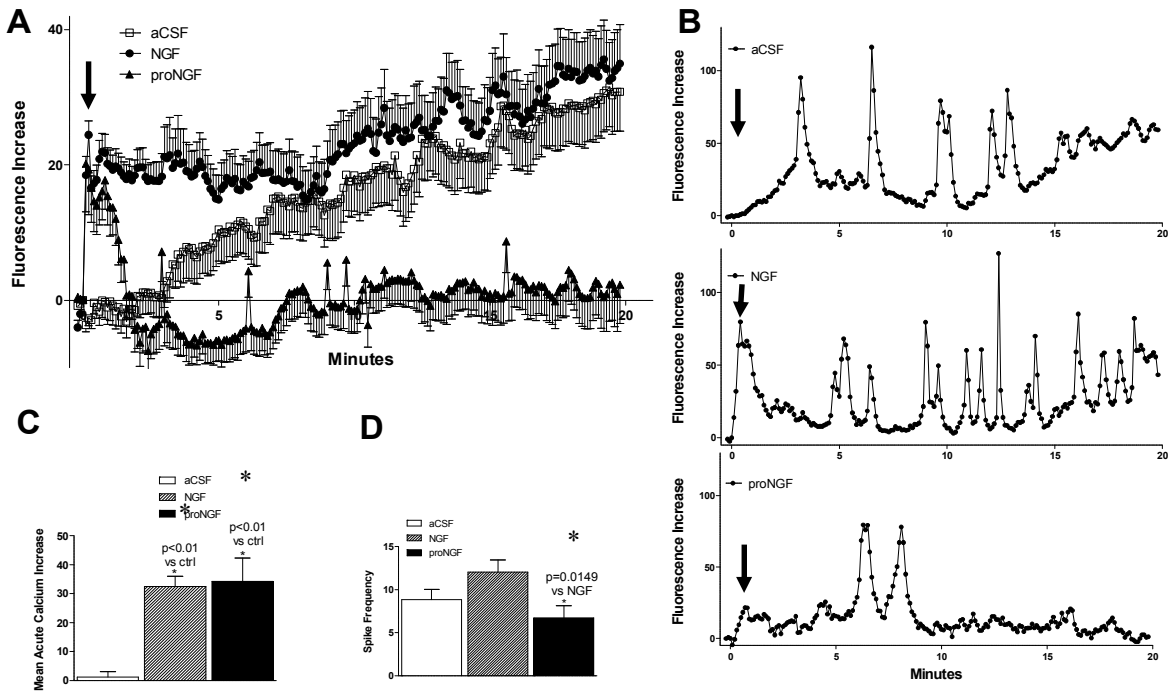


Figure 1.10. Mature NGF and proNGF have different effects on macrophage calcium activity. A. The average intracellular calcium in hMDM following stimulation with aCSF gradually increased in the absence of any acute rise. Incubation of hMDM with 100ng/ml NGF induced a small acute calcium response followed by a gradual accumulation over time that paralleled the accumulation in controls. In contrast, incubation of hMDM with1ng/ml proNGF induced an acute calcium response similar to NGF but failed to stimulate calcium accumulation over time. B. Calcium traces for individual cells illustrating representative patterns of calcium spikes and acute calcium rises (arrows) for control, 100ng/ml NGF and 1ng/ml proNGF treated hMDMs. C. 100ng/ml NGF and 1ng/ml proNGF treatment of hMDMs induced an acute calcium rise after stimulation. D. The frequency of spikes in the hMDM was increased by NGF relative to proNGF.

Effects of NGF and proNGF on protein secretion

Macrophages secrete a wide array of proteins in response to external cues that reflect the diversity of responses of these cells. To understand the full range of the secretory profiles, macrophages were stimulated with NGF or proNGF for 3 days to allow accumulation of proteins in the macrophage conditioned medium (MCM). The medium was then analyzed in duplicate for cytokine, chemokine and growth factor content using the Ray Biotech L-Series Human Antibody Array L-507. Medium from cells challenged with NGF or proNGF were compared to the untreated MCM and to each other to assess individual effects and potential differences between the two peptides. Pre-treatment and post-treatment samples were tightly matched with correlations of protein content ranging from r=0.982 to r= 0.995 indicating that most proteins did not change. Using the 99.8% confidence limit as a cutoff, 9% (46 out of 507 proteins) of the proteins were significantly changed by proNGF and 10% (53 out of 507) significantly changed by NGF. A summary of proteins changed by NGF or proNGF is provided in Table 1.1. Stimulation of secretion was twice as likely as suppression suggesting preferential activation of secretory pathways by each peptide. While the number of positive proteins was similar, NGF and proNGF induced different secretory patterns from the cells with only 10 proteins overlapping. Of these ten proteins, five (GDF, HB-EGF, CXCR2, CCR9, CCR7) changed in opposite directions leaving five for which NGF and proNGF produced similar effects. Four proteins were increased by NGF and proNGF compared to controls (GCSF-R, IL-17F, IL-29, MIP-1β). One protein, IL-13, was decreased compared to controls for both proNGF and NGF, although the suppression was greater for proNGF.

To understand the differences in the secretory profiles between NGF and proNGF, proteins were segregated into categories by function using the Database for Annotation, Visualization and Integrated Discovery (DAVID version 6.7[188,189], a bioinformatics tool. Six major functional groups were identified with the following Benjamin scores reflecting strength of association: response to injury (4.8×10^{-13}) > inflammatory response (1.4×10^{-9}) >, chemotaxis (1.1×10^{-6}), secretion (4.1×10^{-6}) > immune regulation (8.5×10^{-4}), cytokine production (2.0×10^{-4}) and angiogenesis (7.1×10^{-3}). Significant "hits" for categories such as cytokine production would be expected due to the nature of the array, (cytokine panel) but the very high association seen for response to injury and chemotaxis indicated a close relationship between the neurotrophins and these functions. In addition to the above categories, it is worth noting that DAVID also identified bone processes as a significant functional group for NGF but not proNGF. Conversely, adhesion/virus-associated processes were a relevant functional group for proNGF but not NGF.

Although similar functional groups were identified by DAVID for both NGF and proNGF, the minimal overlap of the proteins indicated that the pro and mature forms were regulating the pathways in different ways. To provide a more detailed examination of the patterns of protein secretion after NGF or proNGF stimulation, the positive proteins were grouped into four major clusters (growth factors, cytokines, chemokines and other proteins) with up to seven functional families within each group. Of the four major clusters, growth factors were most highly represented (40/98; 40.8%) followed by cytokines (28/98; 28.6%), chemokines (19/98; 19.4%) and other proteins (11/98; 11.2%). Within the growth factor cluster, seven families were assessed (neurotrophic factors, EGF, TGF, FGF, CSF, IGF and angiogenic factors). NGF and proNGF induced very different growth factor secretory

profiles, summarized in Table 1.1. Most of the time a growth factor was altered by one but not the other peptide. The most notable effects of NGF, illustrated in Figure 1.11, were on proteins within the TGF family where the largest increases were seen in TGF-β3, GDF11, neurturin and follistatin and decreases in GDF3, GDF5 and GDF8. ProNGF increased GDF5, chordin-like 2 and GDF9 and decreased BMPR-IA and GASP-1. Other notable changes (Table 1.1) included increases in FGF-18, FGF-19, IGFI soluble receptor and ErbB2. In contrast, the most notable changes following stimulation with proNGF were within the family of angiogenic factors illustrated in Figure 1.12. Increases were seen in factors associated with suppression of angiogenesis: thrombospondin, thrombospondin-1, angiostatin. Decreases were seen in factors that promote angiogenesis: angiopoieitin, angiopoietin-like 1. No angiogenic proteins were significantly changed with NGF stimulation. Overall, this pattern suggested that proNGF stimulation of hMDMs induced an anti-angiogenic environment whereas NGF may regulate a variety of growth functions.

Chemokine secretion (Table 1.1) was also differentially regulated by NGF and proNGF. Opposing effects (NGF-induced increases, proNGF-induced decreases) were seen for CXCL16, CCR9, CXCR2 and CCR7. Increases in MCP-1, CXCL14, MCP-3, CCL27, MIP 2 and CCR4 were seen after stimulation with proNGF relative to NGF. NGF and proNGF both increased secretion of MIP-1β and CXCR1.

In the cytokine group (Table 1.1), IL17C, IL-17F, IL-17BR, IL-23, IL-23R, IL-24, IL-26, IL-27, IL-28A and IL-29 were increased by NGF relative to control or proNGF. IL-4R, IL-17F, IL-18R and IL-29 were increased by proNGF. Notably, IL-13 was decreased by both NGF and proNGF while IL-10 was decreased by NGF. Many of these proteins are in the IL-17 family and the changes induced for IL-17 related cytokines by NGF or proNGF are

summarized in Figure 1.13. This pattern suggested that the neurotrophins play a role in the secretion of cytokines that regulate immune responses via IL-17 mediated pathways.

The most notable of other proteins altered by NGF or proNGF (Table 1.1, Fig 1.14) were the MMPs. The relative differences in MMP secretion in response to NGF or proNGF are illustrated in Figure 1.14 where a distinct dichotomy is seen between the actions of NGF versus proNGF. NGF selectively induced the release of MMP-7, MMP-8 and MMP-11 whereas MMP-9 secretion was increased after proNGF stimulation. MMP-1 did not meet the cutoff relative to controls but showed a significant differential increase for NGF relative to proNGF.

Since macrophages are commonly categorized as classically activated (M1) or alternatively activated (M2)[26], we examined markers typically used to distinguish these phenotypes. M1 markers were TNF-α, IL-12p70, Il-6, Il-23 IFN-γ, IL-1α,β and IP10. M2 markers were IL-4, IL-10, IL-13, TGF-β (1, 2, 3 & 5), and MCP-1. Changes in the M1 and M2 cytokines, plotted from high to low, are illustrated in Figure 15. The dashed lines show the confidence limits for positive or negative changes. The pattern of increases and decreases did not reveal a clear inflammatory or anti-inflammatory profile for NGF or proNGF with most cytokines showing no change. The most notable effect of NGF was a decrease in IL-1 (M1) and IL-10 and IL-13 (M2). The only increase was for TGF-β3. Stimulation with proNGF did not affect the M1 cytokines. For the M2 cytokines an increase in MCP-1 and a decrease in IL-13 were the only notable effects.

The lack of a clear M1-like or M2-like effect of NGF and proNGF on inflammatory or anti-inflammatory proteins was paralleled by the absence of acute changes in the activation markers CD16 and CD163 analyzed by flow cytometry (not

shown). However, a small decrease in cells expressing $CD206^{high}$ was seen in monocytes treated with either NGF or proNGF for 1 hour (19.1% in controls to 12.3% NGF and 10.9% proNGF, p=0.03) suggesting a small shift away from the M2 phenotype.

Table 1.1. Soluble protein changes in macrophage conditioned medium in response to NGF or proNGF.

Major Families	SubFamilies	Common Name	Protein Name	ProNGF	NGF
Growth Factors	Neurotrophin	beta- Nerve GrowthFactor	beta-NGF	-1906	11832
		Ciliary neurotrophic factor	CNTF	-18712	-879
		Ciliary neurotrophic factor receptor alpha	CNTF R alpha	-2373	7573
		GDNF family receptor alpha-2	GFR alpha-2	-25026	1454
		GDNF family receptor alpha-3	GFR alpha-3	-5592	6571
		GDNF family receptor alpha-4	GFR alpha-4	4081	9819
		Neurturin	Neurturin	855	9621
	Angiogenic	Angiogenin	Angiogenin	10434	-879
		Angiogenin-1	Angiopoietin-1	-12531	-409
		Angiopoietin-like 1	Angiopoietin-like 1	-70270	2873
		Angiostatin	Angiostatin	37867	36
		Thrombospondin	Thrombospondin (TSP)	22839	-879
		Thrombospondin-1	Thrombospondin-1	32165	-879
		Thrombospondin-2	Thrombospondin-2	-14164	-3587
		Osteoactivin	Osteoactivin / GPNMB	8	15189
	EGF	Receptor tyrosine-protein kinase erbB-2	ErbB2	-803	13109
		Cripto-1	Cripto-1	-966	-11589
		Heparin-binding Epidermal like growth factor	HB-EGF	15410	-10820
		Neuregulin 2	NRG2	14881	-879
		Neuregulin 3	NRG3	26006	5257
		Sensory and motor neuron-derived factor/Neuregulin 1 Isoform	SMDF / NRG1Isoform	385	9711
	TGF	Bone morphogenic protein 3	BMP-3	-856	8762
		Bone morphogenic protein receptor-1A	BMPR-IA / ALK-3	-13242	3412
		Chordin-Like 2	Chordin-Like 2	18113	-317
		Follistatin	Follistatin	151	13139
		Growth/ differentiation factor 3	GDF3	-2856	-20946
		Growth/ differentiation factor 5	GDF5	20785	-20505
		Growth/ differentiation factor 8	GDF8	4214	-10327
		Growth/ differentiation factor 9	GDF9	9003	-3250
		Growth/ differentiation factor 11	GDF11	670	16710
		Transforming growth factor-beta 3	TGF-beta 3	539	9859
		Bone morphogenic protein receptor-1B	BMPR-IB / ALK-6	-1841	7282
	FGF	Fibroblast growth factor receptor 4	FGF R4	-15624	187
		Keratinocyte growth factor	FGF-7 / KGF	1430	-14409
		Fibroblast growth factor-13 1B	FGF-13 1B	-10447	-337
		Fibroblast growth factor-18	FGF-18	1507	28892
		Fibroblast growth factor-19	FGF-19	-4230	11039
		Fibroblast growth factor-11	FGF-11	-5817	-12787
	CSF	Granulocyte colony-stimulating factor	GCSF	14252	-2688
		Granulocyte colony-stimulating factor receptor	G-CSF R / CD 114	16035	9283
	IGF	Insulin-like growth factor 1 Soluble Receptor	IGF-I SR	-649	8704

Table 1.1 continued

Major Families	SubFamilies	Common Name	Protein Name	ProNGF	NGF
Inflammatory	Inflammatory	Interferon-gamma	IFN-gamma	-1563	9178
		Interleukin-1 alpha	IL-1 alpha	7040	-21787
		Interleukin-26	IL-26	-6437	27520
		Interleukin-27	IL-27	-2125	11950
		Soluble glucoprotein 130	sgp130	-566	-10926
		Oncostatin M	OSM	-23091	-910
		Interleukin-1 beta	IL-1 beta	-1625	-10394
	Anti-Inflammatory	Interleukin-4 receptor	IL-4 R	66873	-879
		Interleukin-10	IL-10	4650	-19883
		Interleukin-13	IL-13	-22859	-11407
		Interleukin-13 receptor alpha 1	IL-13 R alpha 1	-1344	10114
		Interleukin-10 receptor alpha	IL-10 R alpha	-771	-9682
		Interleukin-22	IL-22	9719	-1403
		Interleukin-24	IL-24	-1352	34953
		Interleukin-28A	IL-28A	-2606	31042
		Interleukin-29	IL-29	28702	26242
	TNF	Tumor necrosis factor receptor superfamily member 5	CD40 liagand / TNFSF5 / CD154	12839	2302
		Lymphotoxin beta receptor	Lymphotoxin beta R / TNFRSF3	-514	14473
		Tumor necrosis factor ligand superfamily member 13	April	10082	863
	Il-17	Interleukin-17B receptor	IL-17B R	-1742	25671
		Interleukin-17C	IL-17C	-1552	17632
		Interleukin-18-binding protein a	IL-18 BPa	-2343	-12256
		Interleukin-18 receptor alpha	IL-18 R alpha (IL-1 R5)	37642	-4101
		Interleukin-21 receptor	IL-21 R	1289	-13178
		Interleukin-23	IL-23	-6551	6152
		Interleukin-23 receptor	IL-23 R	143	22000
		Galectin-4	Galectin-4	-18362	0
		Interleukin-17F	IL-17F	10426	17047
Chemokines	Chemokines	Interleukin-8	IL-8	-10328	-879
		Monocyte Chemoattractant Protein-1	MCP-1	12636	-879
		Monocyte Chemoattractant Protein-3	MCP-3	18210	-620
		Macrophage inflammatory protein-1b	MIP-1b	24631	13195
		Macrophage inflammatory protein-2	MIP 2	34862	-514
		C-C chemokine receptor type 4	CCR4	39880	1276
		C-C chemokine receptor type 7	CCR7	-36858	25721
		C-C chemokine receptor type 9	CCR9	-25369	12536
		C-C chemokine ligand 27	CTACK / CCL27	25312	-879
		C-X-C chemokine ligand 14	CXCL14 / BRAK	12268	-2114
		C-X-C chemokine ligand 16	CXCL16	-16041	8315
		C-X-C chemokine receptor type 1	CXCR1 / IL-8 RA	25821	7695
		C-X-C chemokine receptor type 2	CXCR2 / IL-8 RB	-32262	27759
		C-X-C chemokine receptor type 6	CXCR6	524	13340
		C-X-C chemokine ligand 1	GRO	3051	-13418
		Human CC chemokine-4	HCC-4 / CCL16	-2532	-16397
		Lymphotactin	Lymphotactin / XCL1	-1283	-13629
		Monokine induced by gamma interferon	MIG	-14265	-879
		C-X-C chemokine ligand 1	GRO-a	704	9793
Other	MMP	Matrix metalloproteinase-7	MMP-7	350	17410
		Matrix metalloproteinase-8	MMP-8	-2563	10171
		Matrix metalloproteinase-9	MMP-9	31915	-879
		Matrix metalloproteinase-11	MMP-11 (Stromelysin-3)	-3399	9606
		Matrix metalloproteinase-1	MMP-1	-965	8110
	Adhesion	Intercellular Adhesion Molecule-1	ICAM-1	9536	-2207
		Sialic acid-binding Ig-like lectin-9	Siglec-9	-7361	-16393
	Misc.	Leukemia inhibitory factor receptor alpha	LIF R alpha	-4998	5291
		Inhibin B	Inhibin B	-443	15725
		Growth and differentiation factor-associated serum protein 1	GASP-1 / WFIKKNRP	-12014	1903

Shaded values represent significant changes in protein secretion by NGF or proNGF relative to matched control medium. Values are expressed as relative fluorescence units. Nine proteins showed strong differentials for NGF versus proNGF although no individual changes were seen relative to control (unshaded).

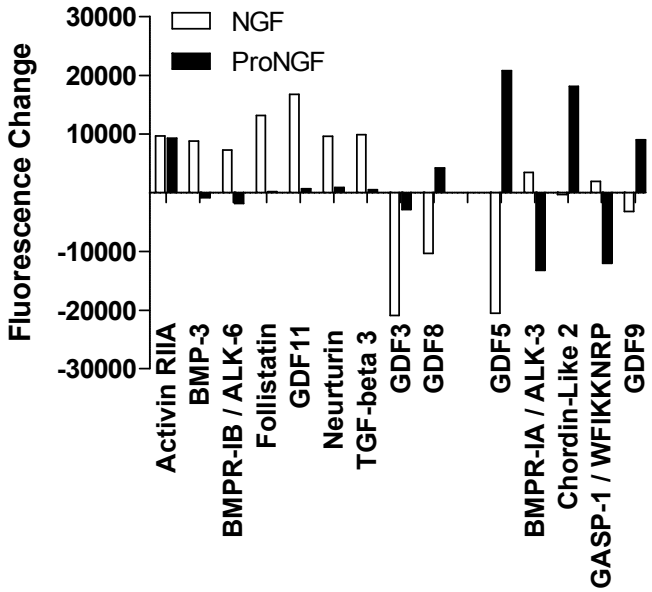


Figure 1.11. Changes in TGF-β family proteins in macrophage conditioned medium induced by NGF or proNGF. Values represent the change in fluorescence signal intensity on the protein array for NGF or proNGF treated cells relative to matched control medium collected prior to stimulation. Differential effects of 100ng/ml NGF and 1ng/ml proNGF were seen for most proteins. Significance limit for the difference scores was $\pm$9207 fluorescence units.

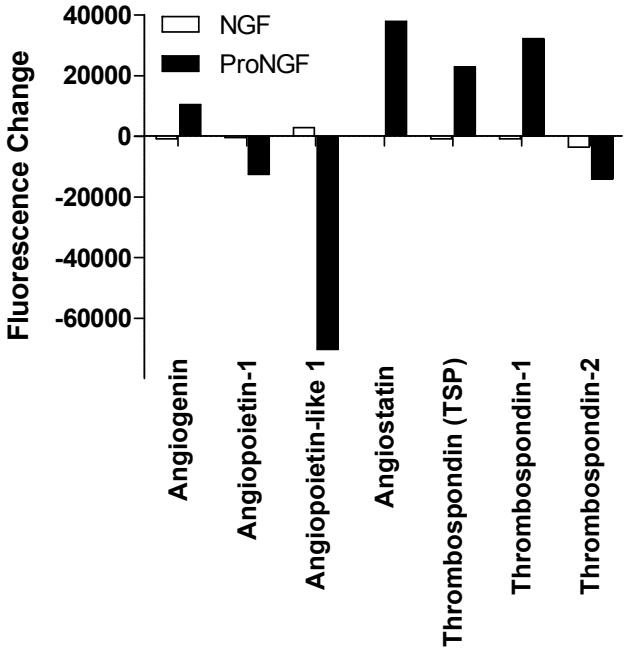


Figure 1.12. Changes in angiogenesis family proteins in macrophage conditioned medium induced by NGF or proNGF. Values represent the change in fluorescence signal intensity on the protein array for 100ng/ml NGF or 1ng/ml proNGF treated cells relative to matched control medium collected prior to stimulation. Only proNGF induced changes in this family of proteins. Significance limit for the difference scores was $\pm$9207 fluorescence units.

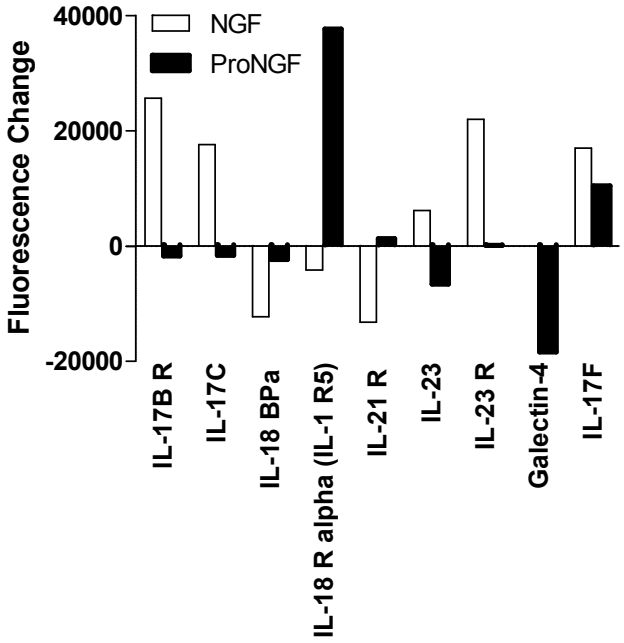


Figure 1.13. Changes in IL-17 family proteins in macrophage conditioned medium induced by NGF or proNGF. Values represent the change in fluorescence signal intensity on the protein array for 100ng/ml NGF or 1ng/ml proNGF treated cells relative to matched control medium collected prior to stimulation. Differential effects of NGF and proNGF were seen for most proteins except IL-17F where both NGF and proNGF increased expression. IL-23 showed a large differential between NGF vs. proNGF but individually was not significant relative to controls. Significance limit for the difference scores was $\pm$9207 fluorescence units.

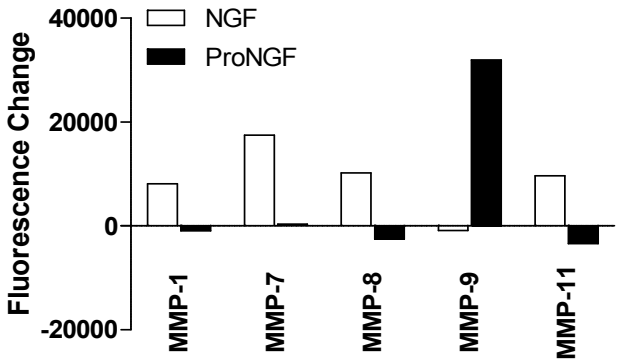


Figure 1.14. Changes in MMP family proteins in macrophage conditioned medium induced by NGF or proNGF. Values represent the change in fluorescence signal intensity on the protein array for 100ng/ml NGF or 1ng/ml proNGF treated cells relative to matched control medium collected prior to stimulation. NGF selectively increased MMP-7, 8, and 11 whereas proNGF induced a selective increase in MMP-9. MMP-1 showed a significant differential relative to proNGF. Significance limit for the difference scores was $\pm$9207 fluorescence units.

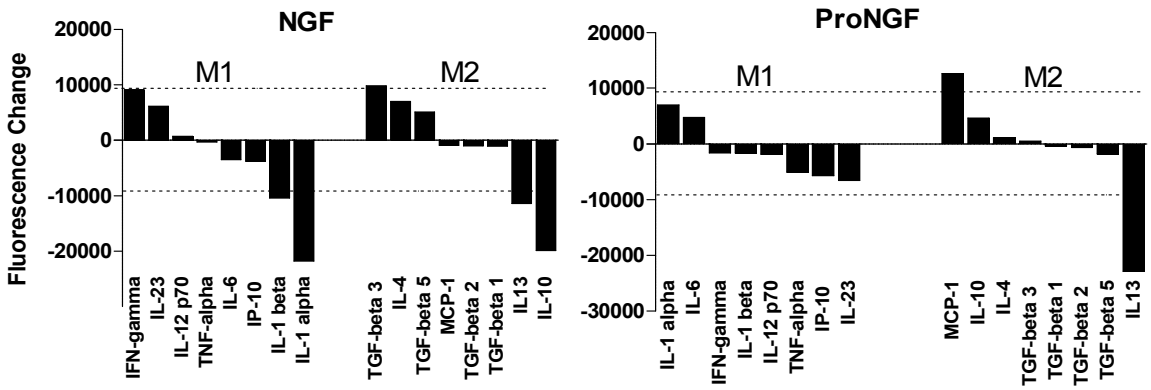


Figure 1.15. Release profile of proteins typically associated with M1 (inflammatory) or M2 (anti-inflammatory) macrophage polarization in response to NGF or proNGF. The dashed line represents the magnitude of change needed to be considered significant. 100ng/ml NGF suppressed the release of several proteins (IL-1α,β, IL-10 and IL-13) and increased TGF-β3. 1ng/ml ProNGF affected the release of only two proteins, an increase in MCP-1 and a decrease in IL-13. Overall, there was no strong trend toward either an M1 or M2 phenotype. Significance limit for the difference scores was $\pm$9207 fluorescence units (dashed lines).

Neurotoxic activity of the hMDM

A prominent response of macrophages to inflammatory stimuli is the secretion of factors that are toxic to neurons [191-196]. Since neurotrophins are prominent in the nervous system we evaluated the ability of the neurotrophins to modulate toxic activity in the macrophage conditioned medium (MCM). Cells were stimulated with NGF or proNGF for 30 min, washed and medium from the conditioned cells was collected after 1 or 24 hours. Toxicity of the MCM was tested on rat cortical/hippocampal neurons pre-loaded with the calcium indicator dye Fluo-4. Previous studies have shown that macrophages activated by inflammatory stimuli release factors that injure neurons via a destabilization of intracellular calcium [197,198]. The effects of the pre-conditioned MCM on calcium regulation are illustrated in Figure 1.16. MCM from hMDM pre-treated with proNGF resulted in a large acute calcium rise followed by partial recovery and then a delayed calcium increase which correlated with the appearance of cell damage in the form of focal varicosities. MCM from cells pre-treated for 30 minutes with NGF showed a larger acute calcium response but no significant delayed rise relative to untreated hMDM. Conditioned medium collected 24 hours after a 30 minute neurotrophin pre-treatment showed negligible calcium responses for both NGF and proNGF (2.2-11.3% of the maximum rise seen in the 1 hour samples) indicating that the toxic effect of proNGF decays over time. Removal of the proNGF was necessary for the decay of activity since continuous treatment with proNGF for 24 hour resulted in similar toxic effects. Medium collected after prolonged stimulation with NGF showed some toxic activity albeit at a reduced level relative to proNGF. Thus, proNGF provoked the secretion of neurotoxic substances from the macrophages whereas NGF had little effect.

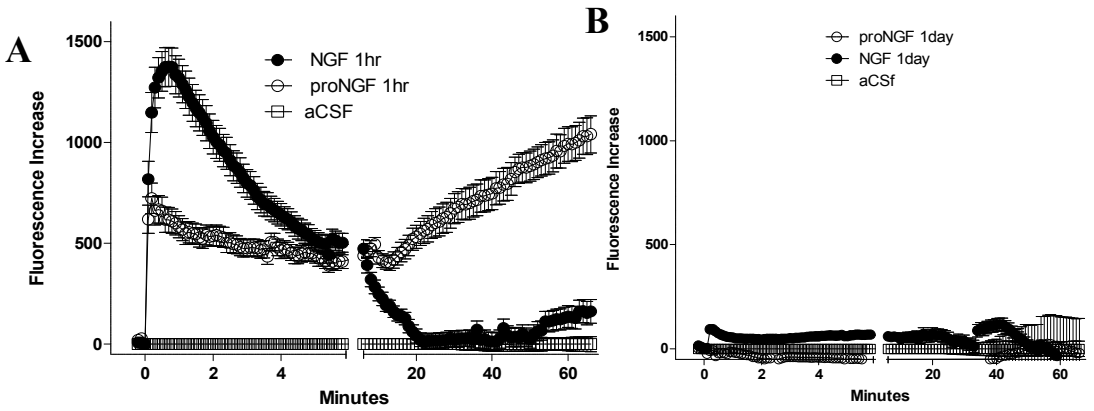


Figure 1.16. Mature and proNGF have opposing effects on the secretion of neurotoxic factors by hMDM. Toxic effects on calcium regulation were monitored by measuring acute (every 6 s for 6 min) and delayed changes in intracellular calcium (every min for 60 min). A-B. Conditioned medium from hMDMs stimulated with 100ng/ml NGF or 1ng/ml proNGF was collected and placed onto cultured rat cortical neurons to assess calcium dysregulation. Macrophages were pretreated with neurotrophins to establish a change in phenotype and then washed and incubated for 1 h in serum free DMEM to generate the macrophage conditioned medium (MCM). A. Addition of MCM at a dilution of 1:5 resulted in an acute increase in calcium for both proNGF and NGF treated cells. NGF caused a larger acute increase compared to proNGF. Delayed accumulation of calcium which correlates with the development of structural pathology was induced by MCM from proNGF treated cells but not NGF-treated cells. Basal calcium levels are illustrated for neurons treated with aCSF. B. Conditioned medium collected 1 day after treatment failed to provoke significant changes in intracellular calcium. Data points are mean $\pm$ sem.

Discussion

Trk and p75^{NTR} expression on hMDM

As early as 1996 Levi-Montalcini introduced the idea that neurotrophins may regulate the immune response[177]. Since this early observation, a few studies have explored neurotrophin receptor gene transcript expression and immunoreactivity in cells of monocyte lineage [169,172,178-184]. These studies showed neurotrophin receptor expression on macrophages but the specific receptor profiles have been variable depending on the tissue and conditions. For example, Hikawa et al. [199] found no Trk receptors in alveolar macrophages whereas Nakajima et al. [178] reported 99% of rat microglia were immunoreactive for all neurotrophin receptors and Garaci et al. [171] found 30% of HIV-infected macrophages were immunoreactive for TrkA and 15% for p75^{NTR}. In our study of hMDM, we found that 47.4% were immunoreactive for p75^{NTR} and 44.3% for TrkA. TrkB was expressed at a much lower level and we observed no significant staining of TrkC. Receptor expression varied from cell to cell suggesting that different phenotypes may be expressed *in vitro* and *in vivo* depending on local influences. Nakajima et al. [178] noted a similar variable neurotrophin receptor expression in rat microglia. However, we also noted that morphological specializations associated with receptor foci were quite labile and changed rapidly in response to various challenges emphasizing the dynamic nature of these features.

We compared the expression levels of neurotrophin receptors on monocytes, the circulating precursors of macrophages, and found that 59% and 29% were positive for p75^{NTR} and TrkA receptors, respectively. High and low p75^{NTR} and TrkA expressing populations of monocytes could be distinguished but separate populations were not evident

for the macrophages. Overall, the expression of $p75^{NTR}$ was not altered after differentiation of the monocytes to macrophages whereas the expression of TrkA may increase.

In neurons, Trk receptors can form homodimers or heteromeric complexes with p75. The strong co-localization of both TrkA and p75 to actin rich membrane specializations in the form of ruffles or finger-like folds on the outer extremities of the cells and the increase in overlapping stained foci induced by NGF indicated that these receptors are poised for interactions. Only a subset of the receptors were precisely co-localized at the membrane folds and within small endosome-like structures in untreated cells but this increased substantially with neurotrophin stimulation. The endosome-like structures were typically positive for either $p75^{NTR}$ or TrkA with a smaller subset positive for both. This pattern was consistent with more detailed studies of endosomal trafficking where $p75^{NTR}$ and TrkA were found to predominantly associate with different endosomes [200]. Although not pursued in the current study, TrkB staining was occasionally seen in these endosome-like structures suggesting some capacity for BDNF signaling in the hMDM.

A large literature on the actions of NGF and proNGF in neurons has highlighted the opposing effects of the pro-form versus mature neurotrophin [185]. Prominent among these effects is the ability of proNGF to induce apoptosis in neurons when $p75^{NTR}$ associates with sortilin [137,201] and the ability of NGF engagement with TrkA/$p75^{NTR}$ to promote survival[121]. The current studies indicated that, like neurons, separate signaling pathways exist for the regulation of innate immune responses.

NGF and proNGF induce different functional macrophage phenotypes

A limited number of studies have identified functional effects of NGF on macrophages. NGF has been shown to increase SDF-dependent migration in human monocytes[202], enhance TNF production in mouse macrophages after lipopolysaccharide (LPS) stimulation[180] and increase macrophage viability during HIV infection[171]. In our study we found that NGF increased the co-localization of TrkA and $p75^{NTR}$ in hMDM, promoted phosphorylation of Akt, and increased survival in serum free medium. NGF also induced ruffling of the macrophages in culture where neurotrophin receptors were found to be enriched. The role of $p75^{NTR}$ and TrkA accumulation at these membrane specializations is not well understood. Patel et al. (2008) suggested that the ruffling of the membrane was associated with phagocytic activity [203]. Indeed, the ruffled cells in our studies showed significantly more phagocytic activity than non-ruffled cells. Other studies in macrophages and microglial cells have shown that increases in intracellular calcium were necessary for cytokine and chemokine release [204] and that calcium spikes, in particular, were correlated with ruffled actin structures and increased phagocytosis [205-207]. In our study, NGF caused an acute calcium rise followed by small random calcium spikes in a subset of cells. A gradual increase in the basal calcium level was also seen in untreated and NGF-treated hMDM that in microglia has been associated with a decrease in LPS-stimulated cytokine secretion[204]. Calcium oscillations have been documented in mouse peritoneal macrophages during attachment and spreading of the cell [206] and in greater than 40% of mouse bone marrow-derived macrophages during phagocytosis[205]. Increases in intracellular calcium are often tightly linked to modifications of the actin cytoskeleton. In the RAW 264.7 mouse macrophage cell line, spontaneous polarization and migration are

associated with influx of extracellular calcium at the leading edge of movement. The calcium increase was necessary for maintenance of F-actin and the ruffled membrane via a PI3K-dependent pathway [208]. Ruffled areas were associated with the accumulation of signaling molecules suggesting that these structures were hotspots for local signaling. In particular these studies suggest that the neurotrophin receptors might be involved in cell migration. In contrast to the leading edge calcium signaling, calcium spikes are thought to be dependent on release of calcium from intracellular stores [205,206] which are necessary for phagocytosis and membrane ruffling. The role of the calcium spikes seen in response to NGF are not known have a potential role in the induction of membrane ruffles and increased phagocytosis.

The effects of proNGF on hMDM function differed substantially from the effects of NGF. No effects were seen on neurotrophin receptor co-localization relative to controls following stimulation with proNGF indicating that the pro-neurotrophin does not promote interactions between $p75^{NTR}$ and TrkA. ProNGF was also closely tied to changes in the cytoskeleton but, in contrast to NGF, induced the expression of podosome-like structures. The podosomes often accumulated in one quadrant of the cell suggesting a polarization that might be associated with migration. This possibility was supported by the transmigration of an increased number of proNGF-treated hMDM across the transwell Matrigel barrier and the lack of an effect on phagocytosis. The calcium response to proNGF also differed from NGF. Although a similar acute rise in calcium was seen, the calcium spikes were greatly reduced relative to NGF and control cells. The reduction was often due to a rundown of spike frequency over time and correlated with the loss of membrane ruffles.

An important effect of proNGF signaling that might be anticipated could be through the interaction of the $p75^{NTR}$ receptor with sortilin a known signaling partner in neurons [201,209]. Only a couple studies have explored sortilin receptor content and function on cells of the mononuclear phagocyte lineage. Sortilin expression was observed on the cell membrane of peritoneal macrophages and bone marrow derived macrophages of rats [210]. Sortilin expression was also found in a microglial cell line, C13NJ. Stimulation of these cells with neurotensin, another endogenous ligand of sortilin, resulted in Akt-dependent extension of actin rich filopodia and migration [211]. We found that sortilin receptors localized to the same ruffled regions as the neurotrophin receptors and the expression of sortilin receptors was increased in the presence of proNGF while NGF slightly decreased its expression. A small proportion of the sortilin receptors co-localized with $p75^{NTR}$. However, this co-localization was increased by both proNGF and mature NGF. In neurons sortilin/$p75^{NTR}$ signaling after engagement with proNGF often leads to apoptosis of the cell [201]. In the macrophages, proNGF did not induce cell death. Unexpectedly, proNGF, like NGF, increased phosphorylation of Akt. A similar effect was seen in microglial cell lines treated with the sortilin ligand neurotensin [211,212]. Although the sortilin-mediated death pathway has been studied extensively in neurons, sortilin participates in other less studied cellular processes such as sorting of proteins in the Golgi compartment [201] and anterograde transport of neurotrophin receptors to enhance neurotrophin signaling [213]. While we cannot rule out a potential role in cell death, our results suggest that sortilin receptors may have alternative functions in macrophages.

Differences in hMDM secretory profiles induced by NGF and proNGF

Secretion of proteins is a primary macrophage function in defense and repair. Of the 507 proteins assessed, approximately 20% changed in response to NGF or proNGF, emphasizing the extensive response diversity of these cells. The ability of NGF and proNGF to activate very different secretory responses indicated that the neurotrophins are capable of shifting macrophage phenotype. In the characterization of the responses, it is notable that the highest scoring functional group using the bioinformatics tool DAVID was response to injury. The next highest scoring groups were inflammatory response, chemotaxis and secretion, all consistent with the structural and functional changes in the cells. Many studies have categorized macrophages into two or three distinct groups based on their secretory profiles and receptor content [26]. Categorizing the response based on classical pro-inflammatory (M1) and anti-inflammatory (M2) cytokines provided little insight into the actions of the neurotrophins as neither peptide induced a clear M1 or M2 secretory profile. We also failed to see changes in surface receptors CD16 or CD163 although NGF stimulation produced a slight downregulation of CD 206, an M2 marker in monocytes. These results along with many other studies have shown that macrophages express a wide variety of phenotypes that extend beyond these two subgroups [214]. Our study in particular indicated that specific protein changes tended to segregate into four alternative functional groups: growth factors, IL-17 family, angiogenic factors and matrix metalloproteases.

The functional group most affected by proNGF and NGF was growth factors. This response highlights the potential importance of neurotrophins in the control of protective and restorative properties of macrophages in disease processes [215]. While macrophages/microglia have previously been shown to secrete neurotrophic factors

[171,178] our profile indicated that other growth factors may be important factors used by these cells. Most prominent were proteins in the TGF-β family including TGF-β3, growth and differentiation factors (GDF3, 5, 8, 9 and 11) and neurturin. Like the neurotrophins, these proteins play a prominent role in growth and differentiation of the nervous system. Most were affected by NGF and the few that were changed by proNGF were invariably in the opposite direction as NGF, again highlighting the opposing actions of the pro and mature forms of the peptide. Little is known about the potential contribution of these growth factors to macrophage and microglial associated tissue protection and should be further explored.

Another family of growth factors that control angiogenesis was almost exclusively affected by proNGF. ProNGF increased angiostatin, thrompospondin and thrombospondin - 1, factors that inhibit angiogenesis while also decreasing factors that promote angiogenesis, angiopoietin and angiopoietin-like 1. Many studies have reported NGF's positive role in angiogenesis [216] however proNGF' s role is less understood. A potential role of the $p75^{NTR}$ has been suggested in some studies. The $p75^{NTR}$ may be particularly important in suppression of tumor metastasis mediated by NGF as $p75^{NTR}$ expression was inversely proportional to formation and metastasis of satellite tumors induced by NGF. [217]. Together these results suggest that macrophages respond to proNGF, perhaps through the $p75^{NTR}$, by the induction of anti-angiogenic activity.

IL-17 cytokines share structural features with NGF [218] and are involved in host defense against extracellular bacterial and fungal infection[219] as well as the pathogenesis of autoimmune inflammatory diseases[220]. The IL-17 family of cytokines was affected predominantly by NGF. With the exception of IL-17F where NGF and proNGF both stimulated an increase, the effects of the two neurotrophins were different. To our

knowledge, no study has examined the effects of NGF on IL-17 levels. However, IL-17 stimulation of human PBMCs upregulates NGF mRNA levels and PBMCs isolated from relapse-remitting multiple sclerosis patients had reduced levels of NGF after IL-17 stimulation [221]. This suggested that IL-17 may influence secretion of NGF and is likely to be a fruitful area which could reveal new insights into regulation of neuroinflammation.

The dichotomy between the actions of NGF versus proNGF persisted for the MMP family of proteins. NGF upregulated secretion of MMP-1, MMP-7, MMP-8, and MMP-11 but had no effect of MMP-9 which was strongly upregulated by proNGF. The secretion of MMPs by macrophages is thought to be mediated in part by podosomes. Since proNGF induced the formation of podosome-like structures in the hMDM, a strong correlation with MMP secretion was expected. MMP 2 and 9 have been localized to podosomes [222]. However, only MMP-9 was increased by proNGF. Secretion of MMP-9 at neuronal synapses is thought to support synaptic plasticity although excess levels can impair long term potentiation (LTP) [223]. Excess secretion in response to proNGF may contribute to the deleterious effects of proNGF in the CNS. Increases in MMP-7 in the CSF have been associated with brain atrophy in HIV-infected patients [158]. In vitro, MMP-7 has been shown to induce atrophy of neuronal synapses via inhibition of vesicle recycling [224]. While this would suggest that MMP-7 secretion in response to NGF is deleterious, a study by Le et al. [156] indicated that the MMP-7 had a protective function during kainic acid-induced seizures by converting proNGF to NGF. More work is needed to clarify the role of macrophage-derived MMP-7. Given the many functions of monocytes and macrophages, the differential regulation of MMPs by NGF and proNGF may have widespread relevance to CNS damage, tumorigenesis, atherosclerosis and other diseases[225,226].

Neurotrophins regulate neurotoxic activity of hMDM

Macrophage infiltration and/or microglial activation often play a key role in the etiology of neuronal damage during neurodegenerative diseases such as Alzheimer and HIV associated dementia (HAD) [227]. The neuropathology has been linked in some cases to the secretion of soluble factors that are toxic to neurons [228,229]. Our understanding of the signaling that gives rise to the pathological actions of macrophages/microglia is poor and considerable effort has been invested to identify therapeutic approaches that control macrophage/microglial activation in neurodegenerative diseases. Changes in the ratio of NGF to proNGF in degenerative diseases such as Alzheimer disease correlate with disease progression and support the idea that a decrease in the ratio of mature, pro-survival neurotrophins to the proneurotrophins is detrimental [230-232]. The different actions of pro- and mature NGF in our studies indicated that the balance of pro- and mature forms may also differentially influence the toxic activity of macrophages/microglia. Our assessment of the neurotoxic activity of macrophage-conditioned medium using calcium homeostasis as a sensitive endpoint indicated that a brief exposure to proNGF induced the secretion of toxic factors. A large delayed calcium dysregulation was seen in neurons treated with MCM which mimicked the toxic activity seen after viral infection of macrophages [197]. Conditioned medium from macrophages stimulated with NGF did not cause the same calcium dysregulation in spite of a similar acute rise in calcium. These observations indicate that the dichotomy between the effects of NGF versus proNGF extend to differences in the ability of the macrophages to exert toxic effects on vulnerable target tissues. These differences have important implications for the control of macrophage activity by

neurotrophins and indicate that $p75^{NTR}$ and TrkA signaling pathways may be fertile ground for the development of strategies to regulate macrophage phenotypes that impact neurodegenerative diseases.

Conclusions

These studies provide an initial characterization of the ability of neurotrophins to influence the phenotype of macrophages. Both NGF and proNGF induce changes in the actin cytoskeleton which favor ruffling of the membranes or formation of podosomes, respectively. Ruffles appear to be a focal point for the expression of $p75^{NTR}$ and TrkA. NGF supported phagocytosis, calcium spiking, ruffle formation, phosphorylation of Akt and the release of growth factors, IL-17 related cytokines and various MMPs. In contrast, proNGF facilitated transmigration, podosome formation and the secretion of anti-angiogenic factors, MMP-9 and factors that induce toxicity in neurons. Like neurons, macrophages are poised to respond to pro- and mature neurotrophins in different ways. These functional differences offer the potential for the development of therapeutic approaches that adjust the balance between beneficial and deleterious macrophage functions.

CHAPTER 3: NEUROTROPHINS MODULATE MACROPHAGE RESPONSES TO HIV

Introduction

Macrophages and microglia play a pivotal role in the development of HIV-associated neurological disease. Soon after HIV infection, activated monocytes infiltrate the central nervous system where they differentiate into perivascular macrophages. These perivascular macrophages are CD16+/CD163+ and harbor a greater viral burden than other cells in brain[30]. It is believed that viral replication by these macrophages spreads to microglia creating a slowly replicating, protected reservoir of virus in the brain leading to chronic inflammatory activation of these cells with an accompanying secretion of neurotoxins [191,192,233]. Much of the current work on these cells strives to identify the macrophage-derived toxins responsible for neural damage and to develop strategies to suppress the toxic activity of the macrophages. This effort is complicated by the fact that macrophages are known to be dynamic cells that can secrete pro-inflammatory cytokines, anti-inflammatory cytokines, enzymes that modify extracellular proteins and/or growth factors depending on their external cues [234]. Thus, an understanding of the impact of the macrophage response to HIV must take into consideration both the positive and negative actions. This is important from a therapeutic perspective since increasing evidence indicates that appropriate interventions may be capable of shifting the balance of activity to decrease damage while at the same time taking advantage of the macrophage's natural ability to protect and repair tissues.

Studies of cultured monocyte-derived macrophages (MDM) in Chapter 2 have shown that MDM robustly expressed neurotrophin receptors TrkA and $p75^{NTR}$ and respond differently to mature nerve growth factor (NGF) and pro-NGF. NGF facilitated a ruffle-bearing phenotype, active calcium signaling and the secretion of proteins associated with growth and repair, and phagocytosis without driving the cells toward a classical M1 or M2 phenotype. In contrast, proNGF favored podosome formation, reduced calcium activity, migration and an anti-angiogenic and neurotoxic secretory profile.

In HIV-infected humans several studies have shown that neurotrophin levels are decreased in brain suggesting a deficit in neurotrophin signaling[235], a scenario similar to other neurodegenerative diseases such as Alzheimer disease where decreases in the ratio of NGF to proNGF correlate with disease progression [230,232,236] [237]. The idea that alterations in neurotrophin signaling may contribute to degeneration in the diseased nervous system is supported by studies showing that neurotrophins and related ligands are protective in models of HIV-associated neuropathogenesis and Alzheimer disease[153,238-241]. While the presumed effects of these treatments were on neurons our demonstration of neurotrophin receptors on macrophages raised the possibility that an alteration in macrophage neurotrophin signaling may contribute significantly to the disease process. Studies have also suggested that NGF may alter CXCR4 expression, the receptor for SDF and co-receptor to HIV but there is conflicting data on the role of NGF in HIV replication in macrophages[171,174,175]. A major function of both the neurotrophin receptors and the chemokine receptors is to modify the cytoskeleton and data in Chapter 2 indicated substantial overlap in the morphological features induced by each. Indeed the known interactions of

HIV with CXCR4 suggested activation of signaling pathways associated with podosome formation that might be similar to the effects of proNGF. Since the polarization of cytoskeletal changes seen with the neurotrophins suggested the possibility for functional overlap with HIV signaling we hypothesized that mature NGF promotes an anti-inflammatory, neuroprotective macrophage phenotype that counterbalances excessive inflammatory activation induced by HIV and/or proNGF. The following studies evaluated the response of macrophages to HIV and potential interactions with neurotrophin receptor stimulation.

Materials and Methods

Isolation and culture of human monocyte-derived macrophages

Human buffy coat leukocytes were purchased and shipped within 24 hours after blood draw from healthy donors at the New York Blood Center (http://nybloodcenter.org/), a non-profit organization specific for the collection and distribution of blood for clinical and research purposes. All research use was screened by the center and no personal identifiers were sent with the shipment. Such use is exempt from review under NIH guidelines, exemption 4 of 46-101(b). Blood was diluted 1:1 with phosphate buffered saline (PBS) and was layered on top of Ficoll-Paque (GE Healthcare 17-1440-03). Blood/Ficoll-plaque was centrifuged at 500 X g for 25 min and the peripheral blood mononuclear cells (PBMCs) were collected from the PBS/Ficoll-Paque interface. PBMCs were washed in red blood cell lysis buffer (Sigma R7757) to remove any red blood cell contamination. PBMCs were centrifuged at 450 X g, the supernatant aspirated and the pellet re-suspended in Dulbecco's modified eagle medium (DMEM) with high glucose, 10% fetal bovine serum (Gibco 160000-044) and 20 μg/ml gentamicin (Gibco 15750-60). Cells were aliquoted into low adhesion 6 well plates

(Corning 3471) at a density of approximately 10^7 cells/well. PBMCs were cultured for 5-7 days to allow monocyte attachment. Remaining white blood cells were washed, from the plate yielding a pure monocyte/macrophage culture. The adherent cells were differentiated into monocyte-derived macrophages (hMDM) using human GM-CSF (15 ng/ml) in complete DMEM for one week. Monocyte experiments were carried out within 1 hour of PBMC isolation to prevent cell attachment.

Primary cultures of rat forebrain

All animal work was done in accordance with NIH animal welfare guidelines and was approved by the University of North Carolina- Chapel Hill Institutional Animal Care and Use Committee board (approval number 14-147.0). Timed gestational embryonic day 9 (E9) pregnant female Long-Evans rats were delivered from Charles Rivers and allowed to rest in UNC animal husbandry until time of experiments. At gestational day E17, rats were sacrificed by anesthetizing with isoflurane drop method until breathing and heart stopped. A thoracotomy was then performed prior to removal of the fetal material. For primary neuronal cultures, fetal material was rinsed in 70% ethanol and placed in HEPES-buffered Hank's balanced salt solution (HBSS) on ice for anesthesia before removal of the brain. The tissue was extensively washed and the cortex/hippocampus was dissected from each brain and cleaned of Dura-arachnoid membrane and visible vessels. The tissue was transferred to a 15 ml tube containing 5 ml calcium-magnesium free-HBSS + 2.4 U/ml dispase + 2 U/ml DNase I and incubated for 25-30 min at 36° C. Tissue was triturated and allowed to settle for 2 min. The suspended cells were transferred to a 50 ml culture tube containing 25 ml of minimum essential medium (MEM) with glutamine + 10% fetal bovine serum + 20 µg/ml gentamicin. After several rounds of trituration in 2-3 ml calcium-magnesium free HBSS, dissociated cells

were seeded at a density 20,000 cells/cm^2 on poly-D-lysine-treated coverslips for imaging and staining or 50,000-100,000 cells/cm^2 in 100 mm plastic dishes for Western blots. After 24 hours, cultures were transferred to Neurobasal medium with B27 supplement. The resulting cultures were >90% neurons at day 4 after seeding.

Immunostaining

Differentiated hMDM grown on poly-D-lysine coated coverslips were transferred to DMEM containing 1% FBS and stimulated for 1 or 24 hours using three different conditions: HIV (10^6virion/ml), HIV +NGF human recombinant protein (100 ng/mL, Sigma N1408), HIV +proNGF human recombinant protein targeted to high affinity sites (1 ng/ml, Alamone N-280), or vehicle (aCSF). The cells were gently washed and fixed in 2% paraformaldehyde in PBS. Cells were washed 3X in PBS and incubated in 3% normal goat serum for one hour. Incubation of primary antibodies was carried out overnight at 4^o C. Cells were stained using antibodies to: $p75^{NTR}$ (Millipore cat #07-476, 1:500), TrkA (Santa Cruz cat #SC-80961, 1:500) and sortilin (Millipore AB9712, 1:500). Cells were washed in PBS and incubated with species specific secondary antibodies conjugated to Alexa 488, 568 or 593 (Molecular Probes) in the dark for 1 hour at room temperature. F-actin was stained using Alexa488 or rhodamine conjugated phalloidin (1:50, Molecular Probes) to show structural changes. Coverslips were then mounted using Fluoromount (Southern Biotech 0100-01) and digitally imaged on an Olympus XI71 microscope.

Analysis of stain intensity for individual cells or regions of interest within the cells was accomplished using Metamorph® software. Co-localization of $p75^{NTR}$ foci with TrkA or sortilin staining was analyzed by staining one receptor red and the other green followed by

thresholding by intensity of stain, computer identification of stained objects and then documentation of each object's central X/Y coordinate for each wavelength. Objects with X/Y coordinates that overlapped within 0.46 microns were scored as co-localized.

Western blots

Human monocyte-derived macrophages were stimulated with HIV for time periods of 0 min, 10 min. At the appropriate time, cells from two 60 mm or one 100 mm dish were harvested using 1.5 ml lysis buffer (1X Ripa buffer (Thermo Scientific 89900), 1:100 phenylmethylsulfonyl fluoride (PMSF, Pierce Chemicals PI 36978), 1:100 Halt® protease inhibitor cocktail (Thermo scientific cat# 1861228). Protein concentration was measured by BCA assay (Thermo Scientific 23225). Protein lysate was resolved on SDS-Page gels (Biorad cat# 456-1034) and transferred to a nitrocellulose membrane (Biorad 162-0112). The membranes were blocked in 2% Bovine Serum Albumin (BSA, Sigma BP1605-100), Odyssey Blocking Buffer (1:1 dilution, Licor 927-40000 for Odyssey) and PBS or milk plus 0.01%Tween (for film) for one hour at room temperature. The primary antibodies were incubated overnight at four degrees. The membranes were then washed in PBS+1% Tween and incubated in secondary antibody for one hour at room temperature. The membranes were then washed again and imaged using the Odyssey or film imaging system. The primary antibodies used were 1:500 $p75^{NTR}$ (Millipore 07-476), 1:500 TrkA (Santa Cruz SC80961). Secondary Antibodies were goat anti-rabbit 680RD (Licor 926-68071) and donkey anti-mouse 800CW (Licor 926-32212), for Odyssey. Film was processed using hoseradish peroxidase (HRP) conjugated secondary antibody and SuperSignal West Pico detection (Thermo Scientific, #34080). .

Phagocytosis of fluorescent beads

Human MDM were stimulated overnight in 1% DMEM with HIV, HIV+ NGF or HIV+proNGF. Fluorescent 1 micron beads (Molecular Probes/Invitrogen F-8887) were placed into each well for 4 hours at a concentration of 4.3 x 10^5 beads/ml. Excess beads not phagocytosed by hMDMs were washed from the plate. The hMDMs were stained with the live cell stain calcein AM (1 μM, 20 min) and digital images of the live cells captured at a magnification of 674X. Some cells were then fixed in 2% paraformaldehyde and stained with phalloidin-Alexa488 (1:50). Cells were individually traced and Metamorph software was used to measure the number of beads in each cell for each condition. The bead density was calculated by dividing the number of beads by the area of the cell. The average bead density was then calculated and compared between the treatment conditions.

Transmigration Assay

Following an overnight stimulation of hMDMs with HIV, NGF+NGF or HIV+proNGF in DMEM with 1% FBS, cells were harvested by incubating in ice-cold calcium-, magnesium-free HBSS for approximately 20-30 minutes to facilitate release of the hMDM from the low adhesion plate. Cells were washed from the plate with a 1 ml Rainin pipette using the flow of medium from the tip of the pipette to dislodge any remaining cells. The hMDM were centrifuged at 80 x g for 5 min, the supernatant carefully aspirated and the soft pellet was re-suspended in DMEM containing 1% FBS. The cells were counted and seeded into 8.0 micron Matrigel invasion chambers (BD Biocoat cat# 354480) in the incubation medium at a density of10^5 cells/chamber. Migratory behavior was measured by the number of cells entering the bottom chamber which also contained DMEM with 1% FBS.

Cells in the bottom chamber were labeled with the fluorescent live cell marker calcein AM (Life Technologies, C3100MP, 1 μM 20 minutes) and counted at 1 and 3 days after seeding.

Flow Cytometry

PBMCs and hMDMs were stimulated with HIV, HIV+NGF or HIV+proNGF in 1% DMEM for 1 hour (PBMCs) or overnight (hMDM). Cells were removed from low adhesion wells and centrifuged for 5 minutes at 450 x g. Cellular pellets were re-suspended and fixed in a Fluorfix solution (Biolegend 420801) for 20 minutes at room temperature. Fixed cells were then treated with permeabilization buffer (EBioscience 020-8333-56) and centrifuged for five minutes at 450 x g at 4º C. The cell pellet was re-suspended in permeabilization buffer and centrifuged at 450 X g at 4º C. Cells were re-suspended in 100 μl of permeabilization buffer plus antibody (1.5 μl p75); Alamone Labs ANT-007-F and 20μl; TrkA R&D Systems FAB1715P; 5μl CD 206 Biolegend 321114; 5μl CD16 Biolegend 302008 and 5μl CD 163 Biolegend 333607) at room temperature for twenty minutes. The stained cells were washed three times in cell staining buffer (Biolegend 420201). Flow cytometry was performed on a FACS Calibur (Becton Dickinson, San Jose, CA) using direct immunofluorescence with at least 100,000 events. Monocytes were determined by populations of cells with high forward scatter and low side scatter. All cells were gated to remove debris. Three color staining analysis was utilized. Cells were analyzed according to side scatter and receptor bound fluorescence, and data was collected with logarithmic amplifiers. Fluorescence spillover compensation was estimated using single-stained and unstained samples with the Cell Quest software (BD). After collection, data was further analyzed with FlowJo software (TreeStar Inc., Ashland, OR).

Protein profiles

Conditions for the analysis of secreted proteins were established to maximize the detection of toxic activity as described. The hMDMs were chronically stimulated for 3 days in 1% DMEM with HIV, HIV+NGF or HIV+proNGF. Medium was collected and centrifuged at 400 X g for 5 minutes to remove any floating cells in the medium. The cell free medium was added to a RayBiotech human antibody array L-507 and processed according to the RayBiotech Biotin Label-based human antibody array protocol. Slide arrays were scanned using an Agilent technologies DNA microarray scanner, and the analysis was carried out using Metamorph software. Internal negative controls were used to establish basal fluorescence and variation across the array. The minimum detectable fluorescence signal was set at 3.2 standard deviation units above the average background to give a probability of 0.001 that a protein signal would be identified as positive by chance. The linearity of signal detection was verified from internal positive standards. Since signal intensity varied between different arrays, protein expression was normalized to the total signal for all proteins on the array and expressed as a relative optical density value to indicate the strength of each signal. Expression of each protein was compared for HIV versus untreated controls. A comparison of HIV to NGF+HIV and proNGF+HIV was then run to determine if the pro and mature peptides interact differently with HIV. Proteins on the array that met the cutoff for a significant change were clustered into functional groups using DAVID software [188,189] and the relative enrichment of various functional classes determined taking into account the focused nature of the array. Based in part on this information, specific functional subgroups were analyzed in greater detail to determine which

classes of proteins were preferentially modified by HIV in the presence or absence of NGF or proNGF.

Neurotoxicity of macrophage-conditioned medium

Pilot studies indicated that a 30 minute stimulation of hMDM was sufficient to induce secretion of toxic factors which persisted for several hours after removal of the stimulus. Thus, the cells could be primed with neurotrophin, washed and the neurotrophin free medium subsequently tested for activity. Macrophages were stimulated with NGF or proNGF in serum free DMEM for 30 minutes. The medium was then replaced with serum free DMEM, the cells were challenged with HIV and the medium collected after 1 hour or 24 hours. The macrophage-conditioned medium (MCM) was centrifuged at 2500 rpm for 10 minutes to remove any cells and then frozen in aliquots at -80^{o} C. The neurotoxic activity of the medium was tested on primary rat neurons cultured on coverslips. Neurons at 6-12 days in vitro were loaded with the calcium indicator, Fluo-4 AM (2 μM, Molecular Probes, Inc., Eugene, OR) in aCSF (aCSF: NaCl 137 mM, KCl 5.0 mM, $CaCl_2$ 2.3 mM, $MgCl_2$ 1.3 mM, glucose 20 mM). After 30 minutes, the coverslip was transferred to a specialized stage for imaging. Cells were maintained in aCSF and time lapse digital images were captured automatically by the Metamorph™ System. Images were captured every 6 seconds for 6 minutes to assess acute effects and every min for 60 minutes to assess delayed effects. Three pre-stimulation measurements were taken to establish basal levels of fluorescence at the beginning of each experiment. Neurons were stimulated with MCM at a 1:5 dilution. The increase in fluorescence intensity within each cell was then measured relative to the baseline fluorescence to correct for cell to cell differences in dye loading and intrinsic fluorescence.

For most studies, cellular responses were averaged across all cells from at least triplicate runs to provide an indication of the "typical" response. In some cases individual cell response patterns are shown where the average masked important cell-specific profiles.

HIV suppresses macrophage calcium activity

Macrophages cultured on coverslips were incubated with 100 ng/ml NGF or 1 ng/ml proNGF and the calcium indicator, Fluo-4 AM in aCSF for 30 minutes. Time lapse digital images were captured automatically every 6 sec for 20 min and changes in fluorescence intensity within each cell measured as described above.

Statistical analyses

Graphpad Prism software was used for data summaries and graphics. Parametric statistics were used to evaluate most changes induced by HIV ±NGF or proNGF. In cases where the data were not normally distributed non-parametric statistics were used. Means were calculated for at least three replicate experiments and the standard error of the mean calculated. T-tests were used for paired comparisons, analysis of variance with repeated measures for temporal data and Chi-square for the analysis of cell populations. The statistical values in the text represent results from t-tests unless otherwise indicated. A probability of <0.05 for rejection of the null hypothesis was considered significant unless otherwise indicated.

Protein array data was analyzed in a stepwise fashion with increasing stringency. First, negative controls on the arrays were used to establish variation within the array. A value 3.2 standard deviation units above and below the mean was calculated and represented the threshold values for a significant increase or decrease. This set the confidence limit at

0.001 so there was only one chance in 1000 that a protein would meet the cutoff by chance. The proteins that were significantly increased or decreased by these criteria were then analyzed using DAVID software to identify functional groups. For comparisons between two conditions, a linear regression analysis was performed to provide an index of how well the arrays matched. This analysis assumed that the majority of proteins on the array would not be changed by the challenge. The data fit this assumption well with all correlations exceeding an r value of 0.9. Proteins that deviated from the linear regression could then be identified on scatter plots as well as by the differential expression between challenge and control conditions. To increase the stringency we then asked how well the changes in protein expression could predict the neurotoxicity of the medium using the maximum delayed increase in intracellular calcium. A cutoff representing strong correlations was set at 2.3 standard deviations above 0 ($p<0.001$) based on the correlation matrix for all proteins across all of the defined functional categories on the array.

Results

p75^{NTR} and TrkA expression in the presence of HIV and neurotrophins

In chapter 2 we identified the expression and localization of neurotrophin receptors p75^{NTR} and TrkA on human monocyte derived macrophages and monocytes. To assess whether HIV could regulate the expression p75^{NTR} and TrkA, monocyte derived macrophages were exposed to HIV virions for 24 hours followed by flow cytometry analysis. Under normal conditions, 53.03 $\pm$8.5% of macrophages expressed p75^{NTR} and 14.1 $\pm$ 1.7% expressed TrkA. Exposure to HIV virions failed to significantly influence the expression of p75^{NTR} (45.2 $\pm$8.5 % of cells,17.3% decrease, p=0.08) or TrkA (11.6 + 2.0% of cells,17.7% decrease). Similarly, 59.2 $\pm$ 14.9% of monocytes expressed the p75^{NTR} and 29.5 $\pm$ 12.6% expressed TrkA. Exposure of HIV virions for 1 hour did not change the expression of p75 and TrkA on monocytes.

Interactions between p75^{NTR} and TrkA have been widely studied in neurons but little is known about their interactions in macrophages. The p75^{NTR} and TrkA are located in overlapping actin rich regions of hMDM (Figure 2.1A, p75^{NTR}-green, TrkA, red, yellow shows overlap). To assess the potential for p75^{NTR} and TrkA to interact in hMDMs, TrkA was precipitated from hMDM protein lysate. Western blot analysis with an antibody specific to the p75^{NTR} identified a 75kDa band in the immunoprecipitated complex confirming that TrkA formed complexes with the p75^{NTR} (Fig 2.1B). This interaction was not changed in hMDMs during exposure to HIV. Flow cytometry analysis confirmed that 71.0% of TrkA expressing hMDMs co-expressed p75^{NTR}. At a cellular level, exposure to HIV for one day did not affect the proportion of cells co-expressing TrkA and p75^{NTR} (65.5% p75 on TrkA). We then sought to determine if regional co-localization of p75^{NTR} and TrkA within

individual hMDMs was altered by HIV exposure. Cultured hMDM were double stained for $p75^{NTR}$ and TrkA, and the overlap of the staining was analyzed at a resolution of 0.46 μm. In the absence of stimulation, an average of 15.9± 2.7% of $p75^{NTR}$ and TrkA foci were overlapping in cells. HIV stimulation increased the overlap of $p75^{NTR}$/TrkA foci to 56.4 ± 0.5% in hMDM (Fig 2.1C; $p<0.001$) indicating that HIV exposure induced a translocation of the receptors into overlapping domains, consistent with data from neurons showing that these receptors normally interact to influence the efficiency of TrkA signaling [118].

In previous studies stimulation of hMDM with NGF greatly increased the co-localization of $p75^{NTR}$ and TrkA. To determine if HIV interacted with the neurotrophins to influence the receptor association we also measured overlap of $p75^{NTR}$+ with TrkA+ foci after co-stimulation with HIV+NGF or HIV+proNGF(Figure 2.1C). NGF+HIV and proNGF+HIV both increased co-localization of $p75^{NTR}$ and TrkA in hMDMs by 132.1% and 343.4%, respectively compared to control cells ($p<0.005$ vs control for both). However, when compared to HIV alone, the overlap was reduced by 34.6% for NGF+HIV ($p=0.006$) and increased 47.7% for proNGF+HIV ($p=0.002$) indicating a small synergistic relationship with proNGF and antagonistic relationship with NGF.

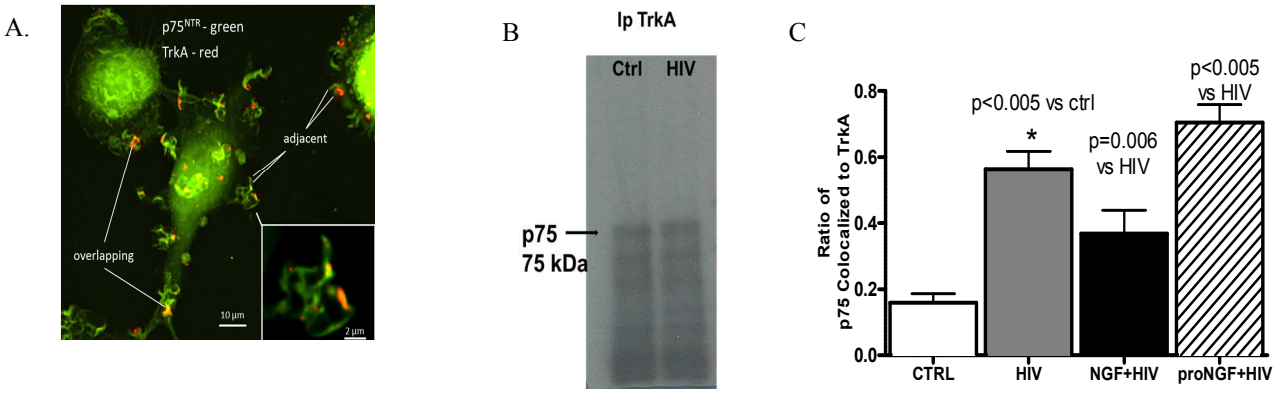


Figure 2.1 .TrkA and $p75^{NTR}$ interactions are increased by HIV in hMDMs. A . A representative picture of $p75^{NTR}$ (green) and TrkA (red) expression on macrophages . $p75^{NTR}$ and TrkA foci are in adjacent and overlapping domains (Inset: yellow regions). B. TrkA was immunoprecipitated from hMDM lysate. Western blot analysis identified a 75kDa band with a $p75^{NTR}$ specific antibody. The $p75^{NTR}$ and TrkA complexes were not changed in hMDM with HIV exposure. Regional overlap of $p75^{NTR}$ and TrkA foci was increased in HIV exposed hMDM. NGF costimulation with HIV reduced TrkA/$p75^{NTR}$ overlap compared to control while proNGF synergistically increased this HIV effect (n=6).

HIV increases p75 and Sortilin co-localization

Because of the relatively robust expression of $p75^{NTR}$ on the macrophages, we examined the expression of other receptors known to partner with $p75^{NTR}$. Sortilin, a well-known signaling partner with $p75^{NTR}$, was also expressed at high to moderate levels in 43% of hMDMs. Sortilin foci were located in similar domains and overlapped 30.7 $\pm$ 0.3% of the $p75^{NTR}$+ foci. HIV had no effect on sortilin expression (Figure 2.2A) but induced a small 39.3% increase in the overlap of $p75^{NTR}$ stained foci with sortilin (Figure 2.2B; $p=0.015$). NGF and proNGF had opposing effects on sortilin expression on hMDM' s exposed to HIV although the magnitude of the changes was quite small. NGF+HIV stimulation increased sortilin expression by 10.16% ($p<0.05$) compared to hMDMs only exposed to HIV. ProNGF+HIV, however, were not different than hMDMs treated with HIV alone. Also the addition of NGF or proNGF to HIV did not significantly affect the increased co-localization of sortilin and $p75^{NTR}$ induced by HIV. This data suggested that although HIV may not have the ability to regulate neurotrophin and sortilin expression, it fosters interactions between $p75^{NTR}$ with signaling partners TrkA and sortilin, creating an optimal environment for potential divergent signaling in response to the natural ligands NGF and proNGF, respectively.

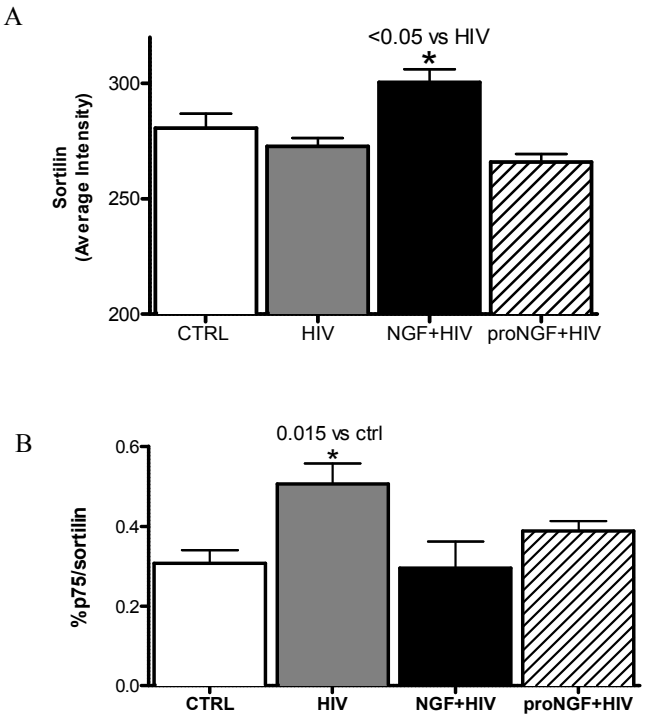


Figure 2.2. HIV increases Sortilin/p75^{NTR} interaction without altering sortilin expression . A HIV and the costimualtion of proNGF +HIV has no effect on sortilin Expression. Co-stimulation with NGF and HIV increases sortilin expression in hMDMs while proNGF. B. HIV increases the p75^{NTR}/sortilin foci overlap in hMDMs. HIV co-stimulation of hMDMs with NGF or proNGF did not affect this p75^{NTR}/sortilin overlap (n=5).

HIV does not trigger death in hMDM

Since signaling of pro-neurotrophins through the $p75^{NTR}$ and sortilin receptor can promote cell death [134,242], we stained hMDMs stimulated with HIV, 100ng/ml NGF+HIV or 1ng/ml proNGF+HIV overnight with the dead cell marker ethidium homodimer and then fixed the cells in 2% paraformaldehyde before staining with the nuclear stain bisbenzimide to identify all nuclei as well as apoptotic nuclei. No difference in dead cells was seen with ethidium bromide staining in any conditions (Figure 2.3; 10-12% loss under all conditions). Condensed and/or fragmented nuclei stained with bisbenzimide were seen in 7-10% of unstimulated MDM or MDM exposed to HIV or NGF+HIV (Figure 2.3B). Cultures co-stimulated with proNGF+HIV showed a small but significant increase to 16% ($p=0.01$ vs HIV). This indicated that HIV does not cause cell death in hMDM but may synergize slightly with proNGF to induce apoptosis in a small proportion of cells (6%).

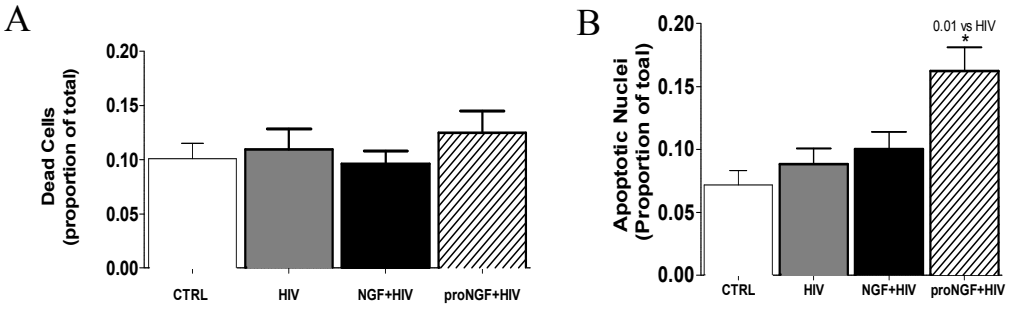


Figure 2.3. HIV does not cause cell death in hMDMs. A .No changes in total cell death in hMDMs stimulated with HIV, 100ng/ml NGF+HIV or 1ng/ml proNGF+HIV overnight with the dead cell marker ethidium homodimer. B. Fixed hMDM stained with bisbenzimide showed no change in the number of fragmented nuclei after exposure to HIV or 100ng/ml NGF+HIV. 1ng/ml proNGF+HIV induced a small increase in the presence of apoptotic nuclei in culture (n=12).

HIV suppresses macrophage calcium activity

Macrophages in culture show a low intrinsic rate of small calcium spike generation. Our data in the previous chapter showed that NGF increased and proNGF suppressed the rate of calcium spiking in the macrophages. HIV interactions with chemokine receptors have also been shown to increase intracellular calcium in cultured human macrophages [103]. The calcium accumulation was due to opening of a non-selective cation channel which was blocked by the CRAC channel blocker, lanthanum. The calcium accumulation triggered the tyrosine phosphorylation of proline rich tyrosine kinase 2 (Pyk2), a protein kinase linked in other studies to the expression of podosomes[243]. Natural chemokine ligands also induced calcium responses but had different effects than gp120 suggesting a unique interaction. However, in these studies the nature of the calcium signaling was not documented. To better understand the effects of HIV on intrinsic and NGF-induced increases in calcium spikes we loaded hMDM with Fluo-4 and measured the frequency of spikes over a 20 min interval. Examples of the patterns of calcium spikes are illustrated in Figure 2.4C as the composite activity of 10 adjacent cells that provide a representative view of the mean responses under each condition. No evidence was seen for synchronization of the spike patterns. Untreated hMDM had an average intrinsic spiking rate of 0.263 ± 0.025/min (Figure 2.4B). This was a slight underestimate of the actual rate in active cells since only 76% of the cells showed significant spikes. Exposure to HIV virions decreased the average spike rate to 0.088 + 0.011/min ($p<0.001$). This was due to both a decrease in the spike frequency and number of cells that showed spiking behavior (46.4%). When HIV was added in the presence of NGF the rate was increased to 0.382 + 0.091 ($p<0.001$). ProNGF+HIV were not significantly

different from HIV alone. Since the calcium spikes were random the patterns were lost in the average data across all cells (Figure 2.4A). The averaged responses showed a small, gradual accumulation of intracellular calcium in vehicle treated (aCSF) hMDM which was also evident in the hMDM treated with HIV+NGF. Stimulation with HIV alone induced a small acute increase in intracellular calcium (arrow) which recovered after approximately 1.5 min. The gradual rise in intracellular calcium was significantly lower than untreated cells. In the presence of HIV+proNGF intracellular calcium decreased over a period of 7-8 min and then increased but failed to reach baseline levels over the 20 min time period. These data indicate that although stimulation of hMDM with HIV results in a small acute influx of calcium, there is a suppression of ongoing calcium spiking. This suppression was reversed by co-treatment with NGF.

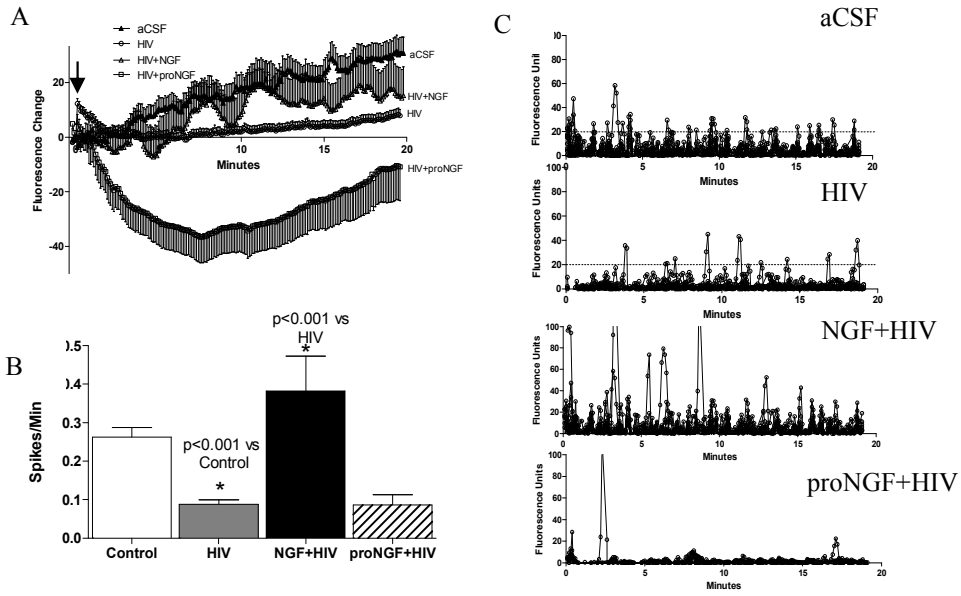


Figure 2.4. HIV and neurotrophins altered the pattern of calcium responses in human monocyte-derived macrophages (hMDM). A. Mean changes in intracellular calcium in hMDM. Treatment with HIV virions suppressed the gradual increase in average intracellular calcium levels seen in vehicle treated hMDM (aCSF). Addition of NGF to the HIV challenged hMDM partially restored the calcium accumulation whereas proNGF with HIV provoked a large decrease in calcium. B. Average spike frequencies within individual cells was greatly suppressed by HIV (and was completely reversed by co-stimulation with NGF (n=10). C. Examples of the spiking patterns for a composite of 10 cells treated with aCSF, HIV, NGF+HIV or proNGF+HIV. HIV decreased the number of spikes reaching the scoring criterion (dashed line). NGF+HIV increased the frequency of spikes whereas proNGF decreased the frequency of spikes.

Modification of macrophage structure

Because of the mobility and diverse phenotypes of macrophages, the regulation of actin structures is extremely important. Findings in Chapter 2 showed that NGF and proNGF induced very different structural phenotypes. To assess changes in actin structures induced by HIV and potential interactions with the neurotrophins, cultured hMDM were stimulated with HIV, NGF+HIV or proNGF+HIV in serum free medium for one hour or overnight , fixed with 2% paraformaldehyde and stained with phallodin-Alexa488 to visualize F-actin. After 1 hr, 37.0 + 5.1% of untreated cells possessed ruffles (Fig 1.8A Ruffled) while 22.9 + 3.0% were rich in podosomes (Fig 1.8A, Podosome). A significant number of cells lacked distinctive specializations (40.1 $\pm$ 4.6%, Fig1.8A, No specializations). The day after medium exchange most untreated cells exhibited specializations with an almost equal mix of cells with ruffles (50.7+0.3%) versus cells with podosomes (46.9+0.3%). HIV stimulation increased the relative number of macrophages possessing podosomes at 1 hour (55.9% increase, $p=0.03$) relative to the untreated cells (Fig 2.5A). Many of the podosome rich cells also exhibited an increased polarization of the podosomes (Fig 1.8A, Polarized) based on a five point scoring system ranging from a uniform distribution of podosomes (1=no polarization) to a large collection of podosomes restricted to one quadrant of the cell (5= high polarization). Untreated macrophages had little polarization (1.32+0.4, Figure 2.5B) while HIV treated cells showed increased polarization (1.78 $\pm$ 0.7, $p<0.001$, 1h).

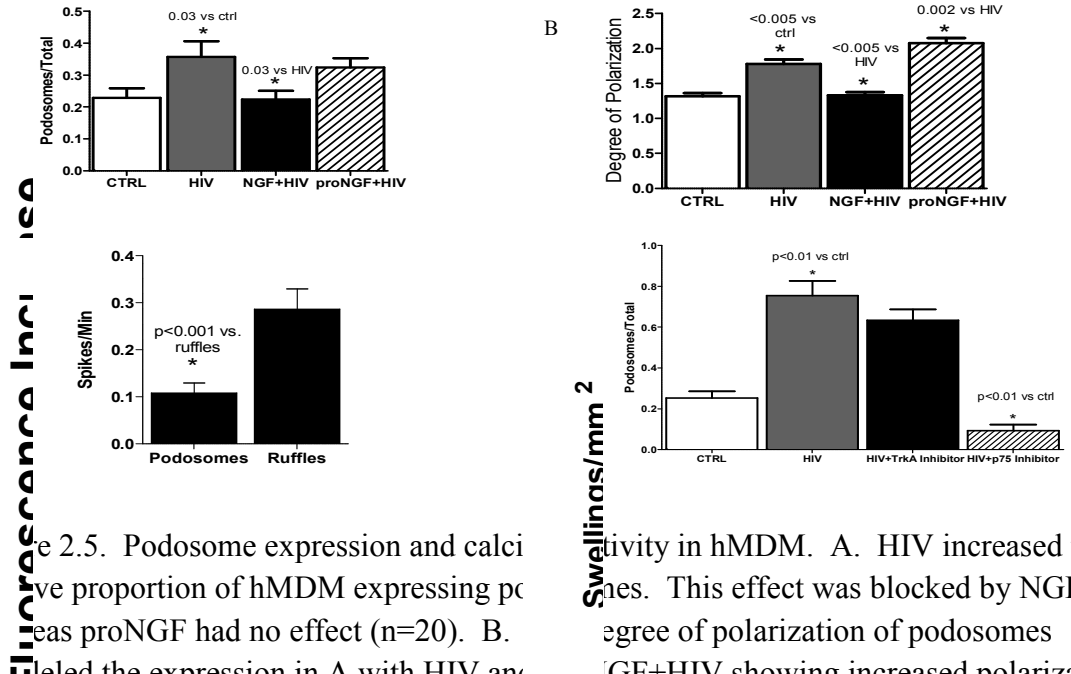
The changes in macrophage actin structures after HIV exposure recapitulated changes previously seen in response to proNGF suggesting the activation of common pathways and the possibility that neurotrophins might modify the response to HIV. We therefore evaluated

the interactions between HIV and mature NGF or proNGF. The addition of NGF to the HIV challenged cells reduced podosome expression to control levels (Figure 2.5A; $p=0.03$). Also, the increased polarization of podosomes by HIV at 1 hour was reversed by treatment with NGF (Figure 2.5B; $p<0.001$). Co-stimulation with proNGF + HIV had no effect on podosomes relative to HIV alone at 1 hour.(Figure 2.5A) but did provoke a small increase the HIV induced degree of polarization by an additional 16.8% over HIV alone (1.7 to 2.1, $p=0.002$; Figure 2.5B). Since the changes in structural phenotypes correlated with changes in the calcium responses, hMDM from eight experiments were segregated into groups by morphological phenotype, (podosome vs. ruffled) and the corresponding calcium activity evaluated. Flat, amoeboid podosome bearing cells showed a suppressed rate of calcium spiking activity relative to cells with ruffles as illustrated in Figure 2.5C. On average ruffled cells displayed 2.6 times the spike frequency of podosome bearing cells 0.287 ± 0.043/min vs. 0.108 ± 0.022/min, $p< 0.001$). In addition, it has been suggested that calcium entry through CRAC channels may activate a signaling pathway associated with the formation of podosomes so we also pretreated the cultures with the CRAC/Trp channel antagonist YM 58483. Overall, there was no significant effect on the proportion of cells expressing podosomes suggesting that the CRAC channel is not involved in HIV induced podosome formation.

Podosome formation is $p75^{NTR}$-dependent

Since HIV and proNGF both increased podosome expression in hMDMs we evaluated neurotrophin receptor involvement in HIV induced podosome formation. Stimulation of hMDM with HIV resulted in an increase in the proportion of cells expressing

podosomes from 0.261 $\pm$ 0.039 to 0.536 + 0.042 (Figure 2.5A) When the $p75^{NTR}$ was blocked with a neutralizing antibody the effect of HIV was not only blocked but the proportion of cells expressing podosomes was reduced below control levels to 0.105 $\pm$ 0.031($p<0.01$). Blockade of TrkA with the specific inhibitor GW441756 did not have a significant effect on HIV-induced podosome expression (0.479 $\pm$ 0.041, Figure 2.5D). These data indicated that HIV induced podosome formation is dependent on p75 signaling and not TrkA.



e 2.5. Podosome expression and calci tivity in hMDM. A. HIV increased the ve proportion of hMDM expressing pc nes. This effect was blocked by NGF eas proNGF had no effect (n=20). B. egree of polarization of podosomes leled the expression in A with HIV anc GF+HIV showing increased polarization)). C. The HIV-induced increase in p ne expression was blocked by inhibition of 75^{NTR} but not TrkA (n=10). D. Measu t of intrinsic calcium spike frequency in expressing ruffles was significantly higher than cells expressing podosomes (n=20).

HIV induces Migration of hMDMs

Stimulation of macrophages with HIV protein, Nef, has been shown to induce podosomes and migratory activity[244]. Stimulation of hMDMs with HIV increased migration across a transmembrane well by 93.2 $\pm$ 0.4% compared to controls ($p=0.013$; Figure 2.6A). Co-stimulation with NGF or proNGF did not have any additional effect on HIV induced migration.

HIV has no effect on phagocytic activity in hMDMs

Phagocytosis is one of the main functions of macrophages and has been correlated with cell ruffling [245]. Since HIV decreased the ruffled phenotype in culture we asked if it also suppressed phagocytic activity. One micron red fluorescent beads were added to macrophage cultures for 4 hours to track phagocytosis. At the end of the 4 hour period cells were stained with the green live cell marker calcein AM. HIV stimulation did not alter the ability of macrophages to phagocytize the beads (Figure 2.6B). However, NGF+HIV reduced phagocytosis by 22.1% compared to controls ($p=0.002$) and by 25.5% compare to HIV alone ($p<0.005$). ProNGF+HIV had no effect on phagocytosis compared to control or HIV-treated cells. This result was counterintuitive since previous studies had shown that NGF shifts the hMDM to a more ruffled phenotype which was associated with phagocytic activity. However, in the presence of HIV we noted that while NGF reduced the number of cells with podosomes a large number of cells lacked both podosomes and ruffles indicating only a partial reversal of the phenotype to an intermediate phenotype with little activity.

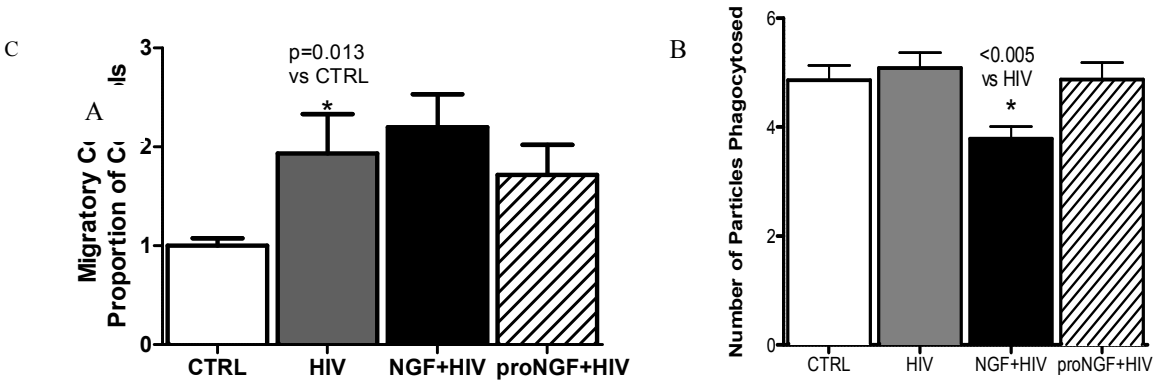


Figure 2.6. HIV increases migration in hMDMs but not phagocytosis. A hMDMs exposed to HIV for one day had increased ability to migrate through a Matrigel barrier compared to untreated cells. The addition of NGF or proNGF did not affect HIV induced migration (n=11). B. Stimulation overnight with HIV ±proNGF had no effect on phagocytic activity of hMDMs. The addition of NGF with HIV decreased phagocytic activity in cultures.

HIV induction of neurotoxin secretion is modulated by neurotrophin signaling

Secretion of unknown neurotoxins from macrophages in response to HIV has been strongly linked to neuronal damage. This neuronal damage includes dysregulation of calcium homeostasis and formation of varicosities along neuronal processes, often referred to as beading (Figure 2.7A). To assess the generation of neurotoxic activity in the macrophage conditioned medium (MCM), hMDMs were stimulated with HIV. Conditioned medium was collected and added to rat cortical neurons at a dilution of 1:5. Calcium accumulation was measured acutely over 6 min followed by assessment of the delayed calcium rise over a period of 60 min. Responses to HIV with or without neurotrophins were compared to control responses in the presence of vehicle (aCSF) or medium collected prior to the challenge. MCM from cells challenged with HIV virions induced a rapid increase in intracellular calcium that recovered partially during the acute phase and was then followed by a prolonged rise in calcium and subsequent formation of varicosities in the delayed phase. The time course of toxin release after stimulation with HIV showed a rapid release peaking at approximately 1 hour followed by a relative stabilization of medium toxicity that lasted for at least 3 days. The rapidity of the response provided the opportunity to pre-condition the hMDM with neurotrophins followed by HIV stimulation and rapid testing of the medium. This was important since any residual neurotrophins would be expected to have direct effects on the neurons. Therefore, hMDMs were pretreated with NGF or proNGF for 30minutes, washed and then stimulated for 1 hour with HIV. When HIV was applied to MDM pretreated with proNGF a significant increase in the toxicity of the MCM was seen based on both the level of calcium destabilization and formation of varicosities(Figures 2.7B and C). In contrast, MCM from cells treated with NGF followed by HIV showed partially reduced

calcium dysregulation and beading. This data indicated that NGF and proNGF have opposing effects on the activation of the hMDM with NGF partially suppressing HIV-induced neurotoxicity while proNGF exacerbated damage.

To understand the role of neurotrophin receptors in the formation of neurotoxin production, TrkA and the $p75^{NTR}$ were blocked with a specific inhibitor, GW 441756, and a neutralizing antibody, respectively, before exposure to HIV virions. A role for endogenous TrkA signaling in the suppression of toxin secretion was indicated by increases in toxic activity after blocking TrkA. Blocking $p75^{NTR}$ with a neutralizing antibody partially reduced toxicity (Figure 2.8A). This data indicated that the activation of the MDM by HIV is influenced by endogenous signaling through TrkA and $p75^{NTR}$.

Since all stimuli that led to the expression of podosomes were also associated with higher levels of toxicity, medium was collected from hMDM predominantly expressing podosomes or ruffles. MCM collected from MDM with ruffles was consistently less toxic than MCM from macrophages with podosomes (Figure 2.8B) providing support for the relationship between podosomes and macrophage toxicity.

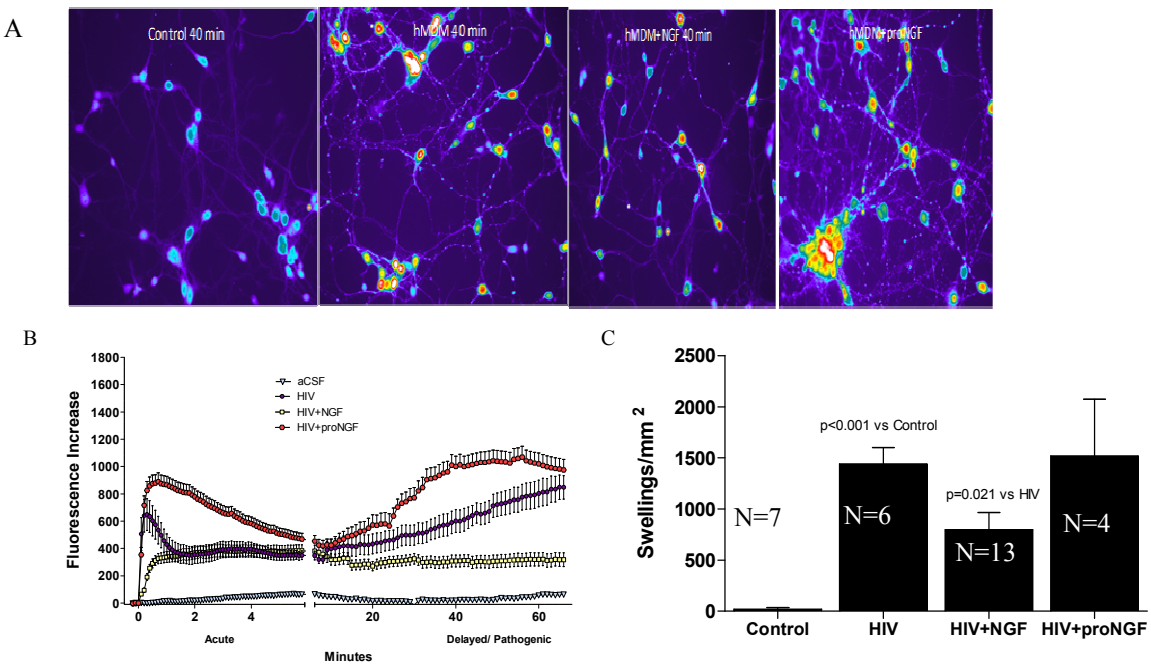


Figure 2.7 HIV increased neurotoxicity is increased by proNGF and partially suppressed by NGF. A. A representation of calcium accumulation and neuronal damage (beading) after 40 minute treatment with conditioned medium from untreated macrophages (Control), HIV (hMDM), HIV+NGF (hMDM+NGF) or HIV+proNGF (hMDM+proNGF). B. Mean calcium accumulation over time illustrating the dysregulation caused by macrophage conditioned medium. Conditioned medium from HIV stimulated macrophages increased calcium influx acutely followed by delayed calcium influx that persisted through the 66 minute experiment. ProNGF increased calcium dysregulation while NGF partially suppressed the delayed rise in calcium. C. Swellings along the dendrites were virtually absent from untreated neurons and dramatically increased by HIV. NGF significantly decreased the number of swellings whereas proNGF had no significant effect (n=6).

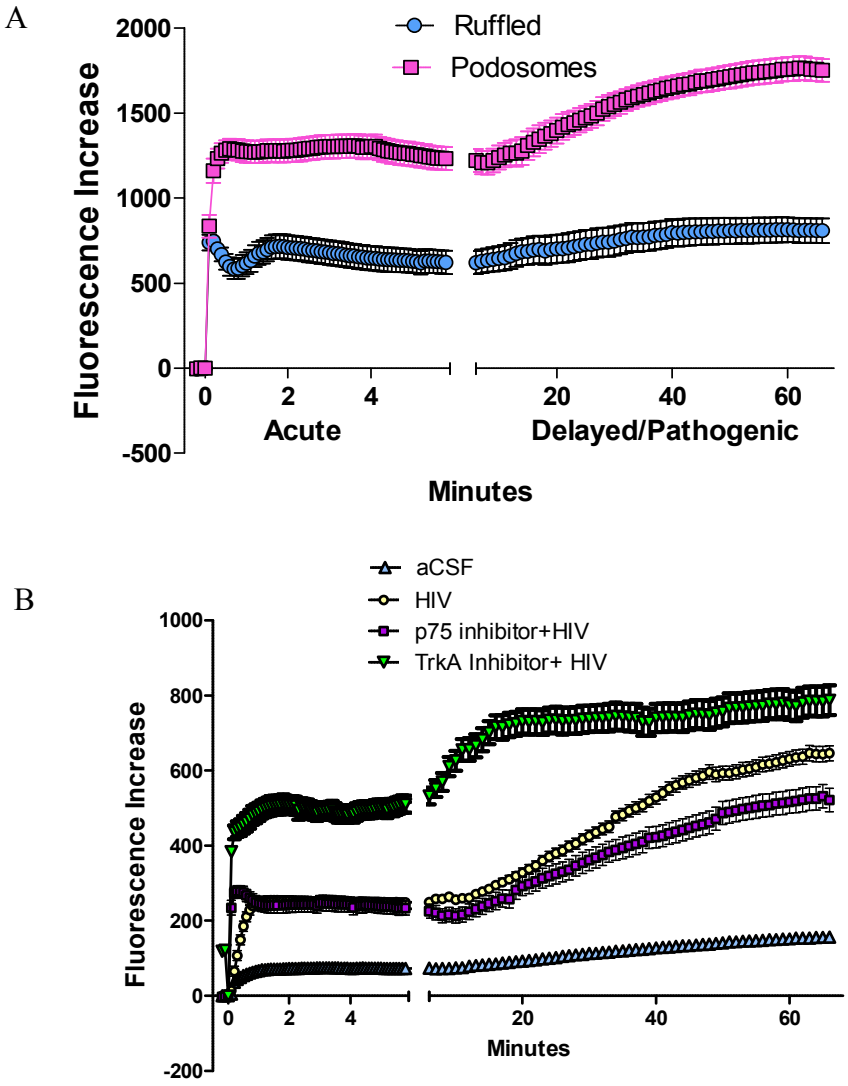


Figure 2.8. TrkA and membrane ruffling blocks meurotoxin formation. A. Graphical depiction of calcium dysregulation caused by macrophage conditioned medium. Conditioned medium from HIV stimulated macrophages increased calcium influx acutely deceasing within 2 minutes, followed by delayed calcium influx that persist through the 60 minute experiment. Inhibition of TrkA signaling with specific inhibitor GW441756 increases neurotoxicity. Blockade of p75 signaling with Ro-08-2750 decreases neurotoxicity. B. Conditioned medium from cultures with mostly podosome bearing cells increases neurotoxin production while cultures with primarily membrane ruffling results in reduced neurotoxicity.

HIV induced a protein secretory profile similar to proNGF which was largely reversed by NGF

Macrophages secrete a wide array of proteins in response to external cues that reflect the diversity of responses of these cells. Secretion of neurotoxins during HIV has been shown to be the main culprit in inducing neuronal damage to the central nervous system. However the neurotoxins causing this damage are unknown. In the previous chapter we showed that NGF and proNGF produced different effects on the macrophage secretome and that the significance of these effects could not be appreciated by a simple analysis of traditional proinflammatory cytokines. To understand the full range of the secretory profile in response to HIV and then to evaluate potential interactions with NGF or proNGF we challenged the hMDM chronically with HIV in the presence or absence of the neurotrophins. The medium was tested on primary neurons to verify toxic activity to ensure that the analysis was relevant to HIV-associated neuropathogenesis. The medium was then analyzed in duplicate for cytokine, chemokine and growth factor content using the Ray Biotech L-Series Human Antibody Array L-507. Medium from cells challenged with HIV, NGF+HIV or proNGF+HIV was compared to untreated MCM to assess the primary effect of HIV and changes in the HIV profile in the presence of NGF or proNGF. Pre-treatment and post-treatment samples were tightly matched with correlations of protein content ranging from r=0.982 to r= 0.995 indicating that most proteins did not change. Using the 99.8% confidence limit as a cutoff, 29.8% (150 out of 503 proteins) of the proteins assessed in this array were significantly changed by HIV, NGF+HIV or proNGF+HIV. A protein was included in the analysis if a significant "hit" was seen in any of the three conditions. A summary of proteins changed by HIV, NGF+HIV or proNGF+HIV relative to controls is

provided in Table 2.1. Changes in the secretory profile of the hMDM in the presence of HIV are illustrated in Figure 2.9. The strong overall correlation (r= 0.901) illustrated the consistency of the arrays with deviations from the line showing proteins that increased (above the line) or decreased (below the line) in the presence of HIV. Notable proteins increased by HIV included MMP-9, TIMP-1, MIP-2, MCP-1, IL-8 and FGF-9. Notable decreases included ICAM-1, angiostatin, and thrombospondin.

Overall, HIV significantly increased the secretion of 30 proteins and decreased secretion of 37 proteins compared to untreated cells. To understand how HIV exposure altered the secretory profiles in hMDMs, proteins were segregated into categories by function using the Database for Annotation, Visualization and Integrated Discovery (DAVID version 6.7[188,189]), a bioinformatics tool. Six major functional groups were identified with the following Benjamini scores, in parentheses, reflecting strength of association: Immune response (8.1E-12), inflammatory response (2.1E-11), defense response (3.5E-11), chemotaxis (5.6E-11), response to wounding (1.8E-10), and regulation of cell proliferation (2.0E-9). DAVID also recognized these functional groupings when evaluating conditioned medium from NGF+HIV and proNGF+HIV stimulated hMDM.

This overlap in functional groupings identified by DAVID did not show the vast differences in protein profiles between HIV stimulated cells and NGF treated cells in particular since it does not discriminate between increases and decreases. To provide a more detailed examination of the patterns of protein secreted after HIV exposure, the significant proteins were grouped into five major clusters that dovetailed with the functional groups identified by the bioinformatics analysis (growth factors, cytokines, chemokines, adhesion/MMP and other proteins) with up to nine functional families within each group.

Within these groups the greatest changes were seen in proteins that regulate angiogenesis, growth, chemotaxis and inflammation.

The most notable changes following stimulation with HIV were within the family of angiogenic factors. Decreases in secretion were seen in factors that promote angiogenesis: angiopoieitin, angiopoietin-like 1 and increases were seen in anti-angiogenesis proteins: thrombospondin and thrombospondin-1, but also suppressed angiostatin and thrombospondin-2. Overall, this pattern indicated that HIV stimulation of hMDMs induced an anti-angiogenic environment.

Other growth factors secreted in response to HIV largely belonged to the TGF and FGF families. HIV increased the secretion of TGF family proteins GDF3, GDF5, GDF15, Chordin like-2 and Activin RIIA but suppressed BMPR-1A and Activin A. In the FGF family, FGF9 and FGF13 1B were increased by HIV exposure while FGFR4, FGF11, and FGF16 were decreased.

Similar to previous reports from cultured hMDM the chemokines MIP-1β, and MCP-1 were increased by HIV [103]. In addition proteins within the MMP family which regulate macrophage invasion and migration were among the more robust changes. HIV increased MMP-9 and tissue inhibitor of metalloprotease-1 (TIMP-1). These changes again indicated that MDM responded to HIV by secreting both damaging proteins such as MMP-9 and protective proteins such as TIMP-1. Thus, there appears to be a constant balance of damaging and protective factors in an attempt to maintain homeostasis.

HIV also increased both inflammatory (IP-10 and IL-17F) and anti-inflammatory cytokines (IL-13). Since macrophages are commonly categorized as classically activated (M1) or alternatively activated (M2) [23], we examined markers typically used to distinguish

these phenotypes. M1 markers were TNF-α, IL-12p70, IL-6, IL-23, IFN–γ, IL-1α,β, and IP-10. M2 markers were IL-4 , IL-10, IL-13, TGF-β (1,2,3&5) and MCP-1. HIV increased M1 marker IP-10 and M2 markers IL-13 and MCP-1. No other proteins classically associated with these activation states were influenced by HIV. Thus, secretory profiles of hMDMs exposed to HIV did not reveal a clear M1 or M2 phenotype. Instead, a combination of inflammatory and anti-inflammatory cytokines was observed highlighting the complexity of the macrophage response.

Neurotrophins reverse HIV induced secretory profiles

In Chapter 2 we saw that macrophage secretory profiles were vastly different after stimulation with proNGF versus NGF. It was therefore anticipated that the presence of HIV might alter these responses in different ways and vice versa. To analyze potential interactions, the HIV secretory response was correlated with the secretory response to HIV+NGF or HIV+proNGF. Strong correlations were seen in both cases (0.951 - 0.956) indicting that changes were restricted to a small subset of proteins. Co-stimulation of macrophages with NGF in the presence of HIV normalized the expression of 58 out of 67 secreted proteins (increased or decreased) by HIV with a synergistic effect seen in only 2 proteins. In contrast proNGF reversed changes in 24 proteins but showed 11 synergistic effects with HIV. To add an additional level of stringency we evaluated the ability of the protein changes to predict the calcium dysregulation seen in neurons under each condition by correlating the protein level to the average maximal delayed calcium level. A cutoff was arbitrarily set at a value that would account for approximately 50% of the variation (r=0.7071). Twenty four proteins met the dual criteria of a response to HIV and a

moderately strong correlation. A summary of the response to HIV, HIV+NGF and HIV+proNGF is illustrated in Figures 2.10 and 2.11. Two response profiles were possible: 1) reversal of an HIV-induced increases or 2) reversal of an HIV-induced decrease in protein secretion and are plotted separately in Figure 2.10 and 2.11, respectively.

The most notable responses in the first group (reversal of HIV increases, Figure 2.10) were MMP-9, TIMP-1, erythropoietin, FGF-9 and IL-4R suggesting modulation of metalloprotease activity and growth. The large effects on MMP-9 and TIMP-1 are particularly notable as MMP-9 has been shown to have deleterious effects on neurons. The normalization of both by NGF is consistent with a protective effect and the increase in MMP-9 with a corresponding decrease in TIMP-1 could explain the synergistic effects of proNGF with HIV stimulation. Other proteins with more moderate changes included IL-17F, CTACK, IL-8, thrombospondin, GDF3, and the insulin receptor. Notable responses in the second group (reversal of HIV decreases, Figure 2.11) included angiostatin, CNTF, FGF-16 and ICAM-1 suggesting normalization of growth and adhesion functions. The potential role of the other proteins is less clear. Overall, these patterns indicate that NGF is a relatively strong antagonist of the effects of HIV, whereas, proNGF has less potent or mixed effects.

In addition to modification of the effects of HIV, NGF in particular also exerted effects that were independent of HIV actions. Twenty proteins changed in response to HIV+NGF and showed high, largely negative, correlations with toxicity indicating the potential modification of toxicity by proteins not linked to HIV activation (Figure 2.12). A striking feature of the response was that, with only one exception, all proteins were decreased by NGF. In addition, a high proportion of the proteins were growth related including most colony stimulating factor proteins (G-CSF R, GM-CSF R, M-CSF, M-CSF R, GDNF, NT-4, IGFBP-3 IGFBP-6,

FGF-7, crypto-1, PDGF-AA and PDGF-BB). This heavy weighting of growth factors indicates a strong role for NGF in the regulation of growth factor secretion. The additional proteins included osteoprotegerin, sgp130, IL-15, IL-19, CCL-16, LFA-1 alpha, HCR, and SCF. An increase in CXCR1 was the only change associated with proNGF reflecting the low overall effect of proNGF on protein secretion. An additional 59 proteins were altered by HIV+NGF and 7 proteins for proNGF, but with low correlations. Notable protein changes in this category were decreases in IL-10, MIP-1alpha, GDF-9, TGF-beta 2, TGF-beta 3, IGFBP-1, MMP-1, and MMP-12 and increases in IL-15, IL-23 and MMP-11. An increase in NGF was also seen, most likely representing the NGF added to the culture.

Table 2.1 Soluble protein changes in macrophage conditioned medium in response to HIV, NGF+HIV or proNGF+HIV

Major Family	Sub Family	Protein Name	Gene Name	HIV minus Control	HIV+NGF minus CTRL	HIV+proNGF minus CTRL
Growth Factors	VEGF	VEGF-B	vegfb	-185	-11909	-304
		VEGF-D	vegfd	-13125	3386	-5559
	Angiogenesis	Angiopoietin-1	angpt1	-17080	-1586	-11899
		Angiopoietin-like 1	angptl1	-49282	8542	-110478
		Angiostatin	plg	-54257	6379	-13720
		Thrombospondin (TSP)	tsp	44047	-9962	42308
		Thrombospondin-1	thbs1	25437	-16953	4236
		Thrombospondin-2	thbs2	-24096	786	-19596
		Osteoactivin / GPNMB	GPNMB	11287	4754	6057
	TGF	Activin A	inhba	-20263	7199	-17269
		Activin RIIA	acvr2a	14199	12185	58291
		BMP-3b / GDF-10	gdf10	-1312	10445	-563
		BMP-4	bmp4	-1106	8198	-1332
		BMP-5	bmp5	-1071	10833	-2172
		BMP-6	bmp6	1236	-11543	1560
		BMPR-IA / ALK-3	BMPR1A	-13149	8409	-12042
		BMPR-II	bmpr2	-2095	34591	-616
		Chordin-Like 2	CHRDL2	21377	7035	48721
		Follistatin-like 1	fstl1	2055	14194	6712
		GDF3	gdf3	37468	11906	28889
		GDF5	gdf5	20089	-23433	17850
		GDF9	gdf9	6574	-24475	3796
		GDF-15	gdf15	38494	7441	12545
		TGF-beta 2	tgfb2	231	-23445	1293
		TGF-beta 3	TGFB3	950	-15079	1422
	EGF	Cripto-1	cr1	-1381	-18412	1129
		HB-EGF	hbegf	7775	-5145	-13674
		NRG2	nrg2	9778	-16420	5278
		NRG3	nrg3	-28587	-16577	-18529
		SMDF / NRG1Isoform	NRg1	936	10643	1788
	PDGF	PDGF R beta	pgdfrb	-1106	-12636	-2312
		PDGF-AA	pdgfa	-379	-23299	520
		PDGF-AB	pdgfb	-6929	-33398	2959
	Neurotrophins	beta-NGF	ngf	-1838	12453	-283
		CNTF	CNTF	-19306	-6085	-44125
		GDNF	gdnf	-681	-12207	-644
		GFR alpha-2	gfra2	-35235	-670	-32253
		GFR alpha-4	gfrA4	-7283	10727	-4795
		NT-4	nt4	-2303	-22062	888
	CSF	G-CSF R / CD 114	CSF3R	-2864	-16524	-2135
		GM-CSF R alpha	gmcsfra	-2609	-12023	424
		M-CSF	mcsf	-1993	-16525	-1785
		M-CSF R	mcsfr	-557	-12217	2940
	IGF	IGFBP-1	igfbp1	1060	-26282	-3869
		IGFBP-3	igfbp3	-599	-17394	72
		IGFBP-6	igfbp5	566	-13172	-62
	FGF	FGF Basic	fgf2	7775	13589	1207
		FGF R4	FGFR4	-49276	-582	-69610
		FGF-7 / KGF	fgf7	-918	-13262	888
		FGF-9	fgf9	74890	-4036	52647
		FGF-11	fgf11	-11998	-10355	-3011
		FGF-13 1B	fgf13	16375	-10825	1045
		FGF-16	fgf16	-14550	21568	1512

Table 2.1 Continued

Major Family	Sub Family	Protein Name	Gene Name	HIV minus Control	HIV+NGF minus CTRL	HIV+proNGF minus CTRL
Cytokine	TNF	CD40 / TNFRSF5	cd40	-11340	5848	-3945
		DR6 / TNFRSF21	tnfrsf21	-3187	-23074	-1360
		GITR / TNFRF18	tnfrf18	-1454	26152	-777
		LIGHT / TNFSF14	tnfsf14	-2541	-13899	6301
		Osteoprotegerin / TNFRSF11B	tnfrsf11b	-149	-18609	1112
		TNF-beta	tnfb	-1789	-25326	-5149
		APRIL	TNFSF13	3396	31518	88043
	inflammatory	IL-1 alpha	il1a	9421	-23446	-2808
		IL-1 F8 / FIL1 eta	il36b	-20017	-4114	-14850
		IL-1 R4 /ST2	st2	-1016	-19799	-807
		IL-1 sRII	il1r2	-545	11245	2855
		IL-26	il26	-5508	32911	-2308
		IL-27	il27	-1658	10586	-668
		IP-10	cxcl10	11265	-9136	499
		OSM	**Osm**	-27554	-709	-19998
		sgp130	**Il6st**	996	-16620	-528
		IFN-alpha / beta R2	ifnar2	-568	15180	170
	Anti-inflammatory	Granzyme A	gzma	26226	-15237	36111
		IL-2 R gamma	il2rg	300	-10766	-1290
		IL-5	il5	4891	30011	4620
		IL-5 R alpha	il5ra	-1767	12712	-88
		IL-7	il7	1493	22820	-2631
		IL-15	il15	10047	28605	-3101
		IL-15 R alpha	il15ra	-19659	4242	-12974
		IL-19	il19	1631	-17579	2047
		IL-22 R	il22r	-18851	238	-5978
		IL-24	il24	-1614	45688	-390
		IL-28A	il28a	-646	22930	-1345
		IL-29	il29	-14217	25263	-9329
		MAC-1	itgam	-12227	-443	-7499
		IL-1 ra	il1rn	-16038	-15624	-23347
		IL-4 R	**il4r**	45779	-11282	84454
		IL-10	**il10**	10058	-24325	4061
		IL-13	**il13**	13340	-21469	-7266
	IL-17	**Galectin-3**	**LGALS4**	-11800	-8186	-20596
		IL-17B R	**IL17rb**	-1165	40417	-4660
		IL-17F	**il17f**	27543	10329	21993
		IL-18 BPa	iL18bp	4288	-14816	1959
		IL-18 R alpha /IL-1 R5	iL18r1	-30292	-2067	-19414
		IL-23	**il23**	-6346	38599	-5799
		IL-23 R	**il23r**	-750	38582	249
Chemokine	Chemokine	**CCL28 / VIC**	**ccl28**	-18558	-12844	4544
		CCR4	**ccr4**	-35225	-8620	-28281
		CCR5	**ccr5**	-1268	10886	-1407
		CCR7	**ccr7**	-39192	10268	-64016
		CCR9	**ccr9**	-32106	182	-60976
		CTACK / CCL27	**ccl27**	32895	-7986	58913
		CXCL14 / BRAK	**cxcl14**	3255	-928	14003
		CXCR1 / IL-8 RA	**cxcr1**	3645	-3979	12469
		CXCR2 / IL-8 RB	**cxcr2**	-43054	13919	-70144
		GRO	**gro**	1617	-21430	-25753
		HCC-4 / CCL16	**ccl16**	-5128	-31160	-3516
		IL-8	**il8**	57102	1737	30797
		I-TAC / CXCL11	cxcl11	-15833	-18464	-4994
		Lymphotactin / XCL1	xcl1	-1151	-23908	266
		MCP-1	ccl2	67321	-27212	22672
		MIG	cxcl9	25792	-15279	693
		MIP-1a	ccl3	-1042	-11729	-400
		MIP-1b	ccl4	14233	-2676	23874
		MIP 2	cxcl2	49392	2112	15954
		PARC / CCL18	ccl18	1308	-15427	358
		Tarc	ccl17	-1837	-14297	-236

Table 2.1 Continued

Major Family	Sub Family	Protein Name	Gene Name	HIV minus Control	HIV+NGF minus CTRL	HIV+proNGF minus CTRL
others	ICAM	ICAM-1	icam1	-72035	-5932	-33392
		ICAM-5	icam5	2057	-6589	-19847
		LFA-1 alpha	itgal	-1644	-11311	-47
		PECAM-1 /CD31	pecam1	-30058	3581	8167
		Siglec-5/CD170	siglec5	-1769	-14842	1588
		Siglec-9	SIGLEC9	-14322	-25798	-13727
	MMP	MMP-1	mmp1	310	-14836	-115
		MMP-9	mmp9	102248	-20867	128646
		MMP-11 /Stromelysin-3	mmp11	-2294	13945	-2748
		MMP-12	mmp12	2499	-23637	4174
		MMP-24 / MT5-MMP	mmp24	16827	-8076	-1368
		TIMP-1	timp1	145157	-12463	65864
	MISC	Artemin	artn	123	35914	-2377
		BIK	bik	-1189	12164	-781
		Coagulation Factor III / Tissue Factor	f3	-10565	4760	-11684
		CRTH-2	ptgdr2	25855	-776	-6070
		EDA-A2	eda	-15802	2885	-11625
		EN-RAGE	s100A12	16991	6437	-1523
		Erythropoietin	epo	127491	-9141	67874
		Frizzled-1	gprasp1	-2030	11373	-356
		GASP-1 / WFIKKNRP	WFIKKN2	-21077	-6537	-20815
		Glut2	slc2a2	819	-14430	1280
		Growth Hormone (GH)	gh1	1398	-14215	-2703
		HCR / CRAM-A/B	hcr	-730	-19280	-612
		Insulin	ins	-3031	16510	-364
		Insulin R	insr	42477	3277	10739
		LIF R alpha	LIFR	10688	-14564	-7603
		MDC	ccl22	-7676	-10891	-8984
		Musk	musk	-9152	-30051	-9911
		Progranulin	grn	19744	-2990	21624
		Prolactin	prl	-10844	-371	-10935
		SCF	kitlg	74	-14389	769
		SIGIRR	sigirr	-16940	-1658	-10231
		SPARC	sparc	9826	-10990	10450
		Thrombopoietin (TPO)	thpo	98	-18007	-1086

Shaded values represent significant changes in protein secretion by HIV, NGF+HIV or proNGF+HIV relative to matched control medium. Values are expressed as relative fluorescence units. Nine proteins showed strong differentials for NGF versus proNGF although no individual changes were seen relative to control (unshaded).

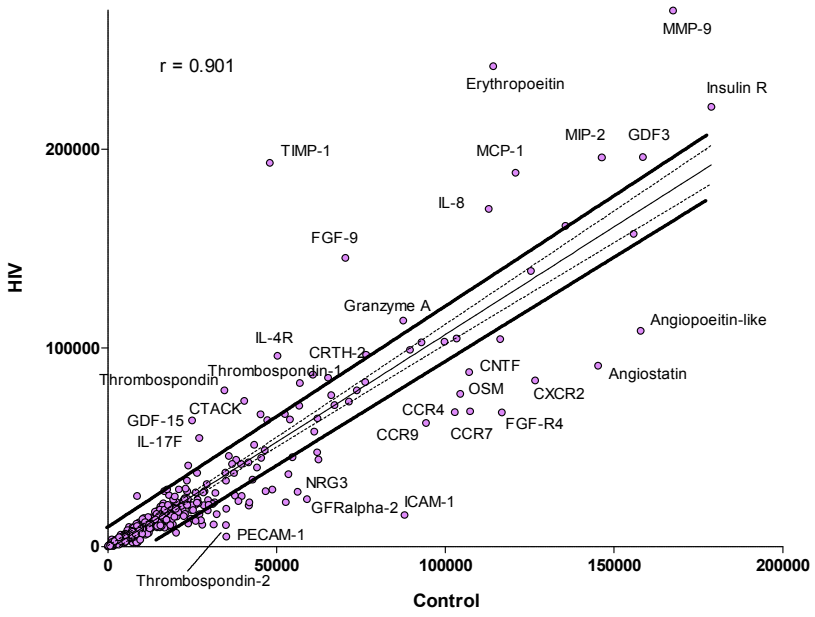


Figure 2.9. Changes in secretory profiles induced by HIV. Values represent the correlation of changes in fluorescence signal intensity on the protein array for HIV versus untreated hMDMs. r=the correlation coefficient. The solid line represents the 99.9% confidence limits.

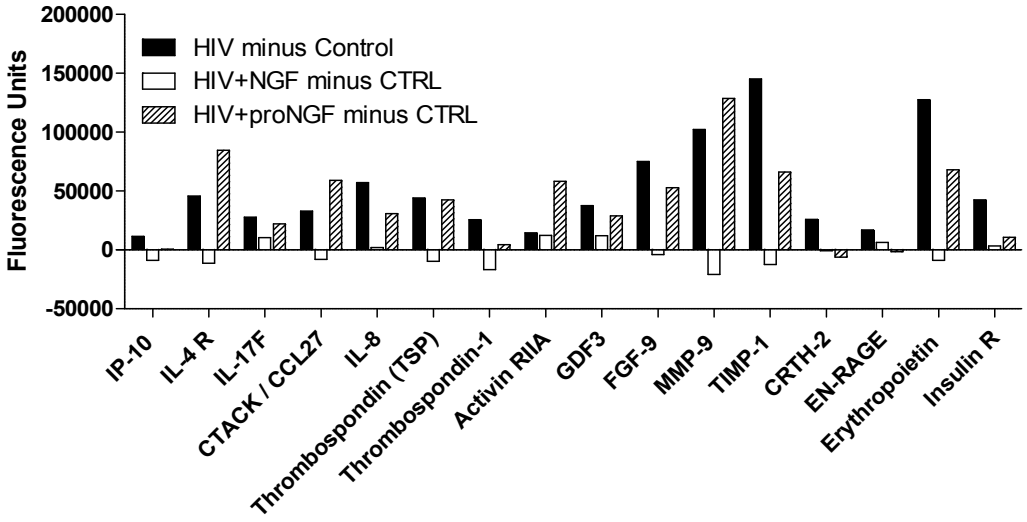


Figure 2.10. Proteins that were increased by HIV and correlated with toxicity. Values represent the change in fluorescence signal intensity on the protein array for HIV, 100ng/ml NGF+HIV or 1ng/ml proNGF+HIV treated cells relative to matched control medium. NGF reversed the HIV-induced increase in almost all cases, largest of which were MMP-9, TIMP-1 and erythoropoietin.

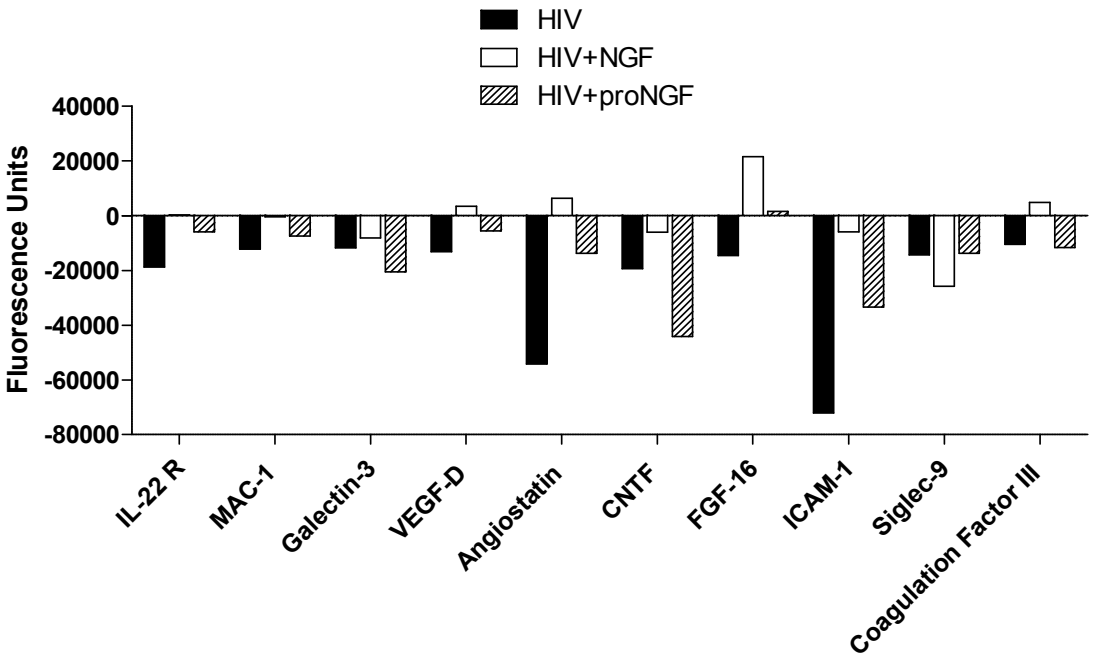


Figure 2.11. Proteins which were decreased by HIV and also correlated with toxicity. Values represent the change in fluorescence signal intensity on the protein array for HIV, 100ng/ml NGF+HIV or 1ng/ml proNGF+HIV treated cells relative to matched control medium collected prior to stimulation. NGF reversed these decreases with only one exception.

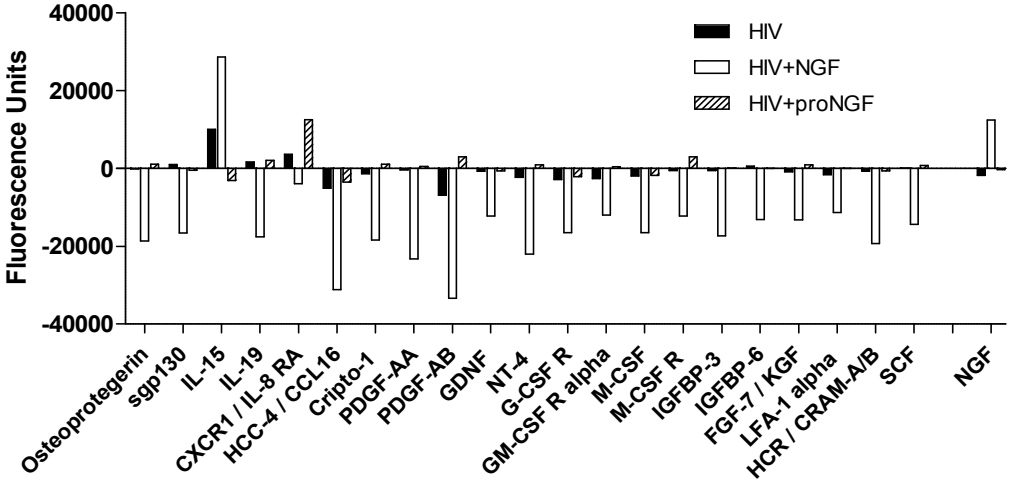


Figure 2.12. Effects of NGF in the absence of significant effects of HIV. Values represent the change in fluorescence signal intensity on the protein array for HIV, 100ng/ml NGF+HIV or 1ng/ml proNGF+HIV treated cells relative to matched control medium collected prior to stimulation. Thirteen of the 22 proteins were growth factors or related proteins and all except IL-15 and NGF decreased in the presence of HIV+NGF.

Analysis of cell surface phenotypic markers

Although we did not identify a classical M1 or M2 phenotype in our secretory analysis we evaluated changes in three cell surface markers that had previously been associated with HIV infection and migration into the CNS, CD16, CD163 and CD206. Expression of CD16, CD163 or CD206 on both monocytes and hMDM was evaluated by flow cytometry and no significant changes in total expression were seen under any of the conditions. However, an analysis of a subset of monocytes expressing high levels of CD206 indicated that HIV induced a small but significant decrease in the $CD206^{high}$ population (-22.7%, p=0.018; Figure). A decrease was also seen with NGF+HIV (-33.4% relative to control, p=0.04) and HIV+proNGF (-60.7%, p=0.006).

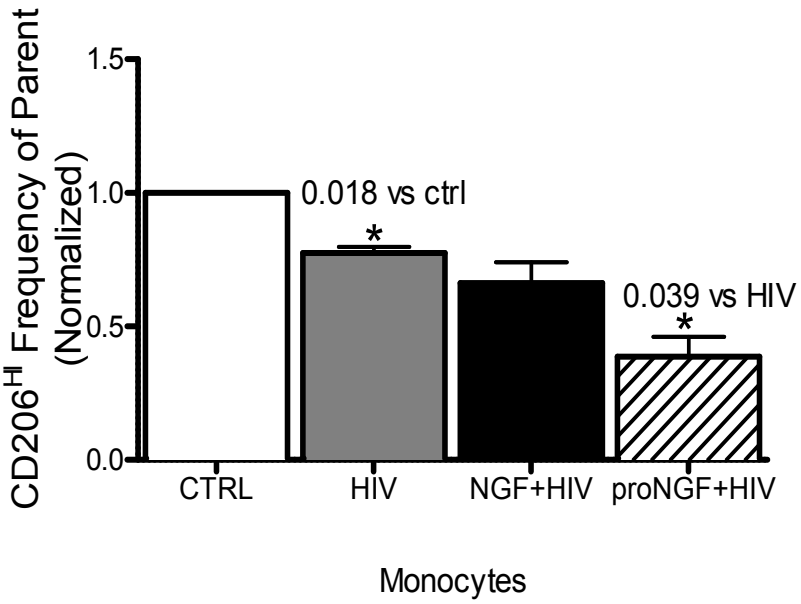


Figure 2.13. HIV decreases the M2 surface marker CD206. A. Stimulation with HIV decreased a subset of $CD206^{high}$ expressing monocytes, a M2 marker which was synergistically decreased by the addition of proNGF(n=3).

A

Discussion

HIV induces a neurotoxic phenotype in hMDM

Macrophage activation is the key correlate of damage severity during HIV neuropathogenesis; however, how this activation leads to toxicity is still not fully understood. This lack of understanding has been a significant hurdle for the development of interventions that control inflammation. In this study we showed that macrophage activation by non-infectious HIV virions induced the expression of more podosome like structures and fewer ruffles. The podosomes were often polarized, accumulating in one quadrant of the cell suggesting a potential role in migration. Podosomes are sites of adhesion thought to facilitate migration through tissues via the release of MMPs. Our previous studies have shown that NGF and proNGF are naturally occurring stimuli that also induce (proNGF) or suppress (NGF) the expression of podosomes. Consistent with this observation, we observed an increase in podosomes when proNGF was added to HIV and a decrease with NGF co-stimulation.

Increases in intracellular calcium are necessary for podosome formation as well as many other cytoskeletal modifications. HIV induced a small acute calcium response but the most prominent effect was a suppression of calcium spiking. Calcium spiking in macrophages has been correlated with membrane ruffling [205-207,246]. The loss in cells containing membrane ruffles during HIV exposure may explain why calcium spiking is suppressed and why we saw no change in phagocytic activity.

Neurotrophins differentially regulate HIV-induced macrophage functions

An imbalance of pro-neurotrophin versus mature neurotrophins in the central nervous system has been implicated in neurodegenerative diseases such as Alzheimer disease [247-

249] and has been measured in the hippocampus of HIV-gp120 transgenic mice [152] . Much of the work assessing the impact of pro and mature neurotrophin imbalance has been done in the context of neuronal damage. However it is now clear that macrophages and microglia are significant targets of the neurotrophins largely due to expression of the $p75^{NTR}$ and TrkA. Although HIV exposure did not influence $p75^{NTR}$ and TrkA expression on hMDMs it increased the co-localization of the receptors suggesting HIV regulation of $p75^{NTR}$ / TrkA signaling. Blocking TrkA signaling enhanced neurotoxin production and the formation of podosomes in response to HIV, while blocking the $p75^{NTR}$ reduced neurotoxin production, possibly through another signaling partner. In fact, our studies showed that HIV induced a modest increase in the co-localization of the $p75^{NTR}$ with another known signaling partner, sortilin. Differential interactions of the $p75^{NTR}$ with TrkA versus sortilin has been widely cited in the literature, in part, to explain the detrimental effects of pro-neurotrophin signaling. The $p75^{NTR}$/TrkA receptor complex engages with NGF and leads to pro-survival signaling events whereas $p75^{NTR}$ /sortilin engage with proNGF and has been associated with cell death. In this current study however no cell death was seen with HIV exposure, suggesting another role for sortilin in hMDMs. HIV's ability to encourage interaction between the $p75^{NTR}$ and both TrkA and sortilin suggests that the cell is primed to interact with both NGF and proNGF with different consequences. Consistent with this possibility, we found that NGF and proNGF exerted opposing effects in almost all assays.

As many of the results seen during HIV in the study overlapped with our previous findings for proNGF we characterized the impact of stimulation with proNGF or NGF during HIV stimulation. The addition of proNGF to HIV treated macrophages did not alter many functions seen during HIV stimulation such as increased co-localization of $p75^{NTR}$ with

TrkA, sortilin expression, suppression of calcium spiking, podosome formation, and phagocytosis. However the addition of proNGF did increase the number of cells with polarized podosomes and the toxicity of the conditioned medium. Unlike proNGF, NGF co-stimulation resulted in a vastly different phenotype in HIV treated macrophages. NGF reduced HIV's ability to increase $p75^{NTR}$ and TrkA co-localization. Ironically NGF increased sortilin expression but decreased its overlap with immunopositive $p75^{NTR}$ foci. Importantly, NGF reversed each of the major effects of HIV. Podosome expression and polarization were reduced by increasing the number of cells with no specializations. Calcium spikes were increased and we saw a reduction in phagocytic activity. Since phagocytic activity has been linked to calcium spikes this decrease in phagocytic activity was counter intuitive. However phagocytic activity has also been correlated with membrane ruffling and the shift in away from podosomes and ruffles may explain the reduction of phagocytosis and suggest a transitional phenotype caused by NGF and HIV stimulation in macrophages.

HIV and hMDM neurotoxin production

Neuronal damage in HIV infection has been linked to the secretion of soluble factors, or neurotoxins from hMDMs, but the factors that cause neural damage are not well defined. In this study we assessed the secretory profile of hMDM after HIV exposure. Many groups have tried to classify macrophage activation states as inflammatory (M1) or alternative anti-inflammatory (M2). However in our study there were no classical M1 or M2 activation phenotypes seen during HIV exposure although a small down regulation of the M2 surface receptor CD206 was seen. Although cytokines did not match a M1 or M2 phenotype HIV did increase cytokines and chemokines that have previously been shown to be increased by HIV

including MCP-1, MIP1b, and MIP-2. Co-stimulation with NGF and HIV returned these proteins back to control levels indicating an antagonistic relationship between the effects of HIV and NGF. ProNGF addition either had no effect on proteins increased by HIV (MIP-1β) or reduced them (MCP-1, MIP2).

To understand the changes seen in the secretome we utilized DAVID, a bioinformatics tool, which characterized these proteins as being involved in immune response, defense response, chemotaxis response, wound healing and regulation of cell proliferation consistent with known functions of hMDM during interactions with virus. Instead of traditional inflammatory categories, HIV influenced specific proteins that were segregated into four major functional groups: growth factors, cytokine, chemokines, and other proteins.

The family most affected by all stimulations was the growth factor family and highlighted the importance of assessing both beneficial and detrimental properties. Similar to the results in Chapter 2 for proNGF, HIV and co-stimulation with proNGF facilitated the secretion of proteins that favored an anti-angiogenenic environment while co-stimulation of hMDMs with NGF blocked the increased secretion of all anti-angiogenic factors induced by HIV. This is consistent with the breadth of literature implicating NGF's role in promoting angiogenesis [216]. Although little is known about the significance of angiogenesis in HIV infection much of the work done on angiogenesis has been in the context of Kaposi sarcoma, an angioproliferative mesenchymal cancer often seen in AIDS and other immune deficient patients. HIV Tat and nef have been shown to promote Kaposi sarcoma induced angiogenesis[250,251]. Anti-angiogenic factor thromposondin-1 inhibits HIV-Kaposi sarcoma induced angiogenic activity[252]. In our studies, thrombospondin and

thrombospondin -1 were increased with HIV and angiostatin was reduced by HIV suggesting that HIV may have a role in halting angiogenic activity. ProNGF co- stimulation of macrophages with HIV increased the secretion of thrombospondin, thrombospondin-1, and angiostatin. The increase in anti-angiogenic factors induced by proNGF suggests that there may be a role for $p75^{NTR}$ in anti-angiogenesis. This is consistent with a study that showed $P75^{NTR}$ expression was upregulated in endothelial cells of type one diabetic mice and was involved in inhibiting neovascularization [253]. These findings suggested an important role in proNGF signaling in macrophages during cancer prevention including HIV-Kaposi sarcoma .

The TGF and FGF families were most highly represented however not much is known about these families in the context of HIV. The most robust changes came from the GDF subgroup of the TGF family. However the only member of this subgroup that correlated with toxicity was GDF3. Likewise, in the FGF family, FGF-9 was increased and FGF-16 was decreased with HIV. Not much work has been done on FGF-9 or FGF16 in macrophages or in the context of HIV. FGF-9 has been shown to be secreted from neurons and support basal forebrain cholinergic neurons [254]. However, more work needs to be done to understand the importance of these factors in the context of HIV neuropathogenesis.

Few of the results from the protein arrays provided leads that might identify the protein(s) responsible for neurotoxicity. The strongest candidates from these studies were proteins within the MMP family. NGF increased MMP-7 and MMP-11 in the presence and absence of HIV. Support for the importance of the MMPs has come from studies showing their involvement in synaptic damage and remodeling. MMP-7 was low in the medium of unstimulated cells and largely unaffected by HIV, however, it was increased by NGF. MMP-

7 has been shown to be one of the enzymes that cleaves proNGF to NGF [255] and has also been implicated in positive synaptic remodeling by altering presynaptic terminals without affecting neuronal survival [224]. NGF's ability to increase MMP-7 secretion may be a feedback mechanism to support NGF signaling while also aiding in reconstruction of any damage caused to neurons during HIV exposure. A potential positive influence of MMP-7 was supported by the negative correlation with toxicity.

MMP11 secretion was also increased by NGF stimulation. Most of the work looking at MMP-11 in the central nervous system involves gliomas [256,257]. It is unclear if it is involves in HIV neuropathogenesis but the anti-angiogenic phenotypic switch with proNGF stimulation in macrophages found in this thesis may provide a new therapeutic avenue in brain cancer research and should be explored further.

One of the most striking observations from the protein array analysis was the increase in MMP-9 and the tissue inhibitor of metalloprotease-1 (TIMP-1) in response to HIV. Both proteins were robustly expressed and had positive correlations to calcium dysregulation. The increase in both MMP-9 and its endogenous inhibitor TIMP-1 suggested that the hMDM constantly provide potentially deleterious and protective proteins at the same time in an effort to maintain tissue homeostasis. The balance between MMP-9 and TIMP-1 may be a crucial factor in the regulation of tissue damage and has been seen in other brain injury models including ALS [258-261]. Evidence for deleterious effects when the balance is changed was seen in the effects of proNGF which primarily decreased the secretion of TIMP-1, a result that would predict the increase in neurotoxicity of MCM after stimulation with both HIV and proNGF. MMP-9 is involved in activity dependent reorganization of dendritic spines and long term potentiation and has been implicated in abnormalities in spine morphology in a

fragile X syndrome mouse model [223,262,263].MMP-9 has also been associated with neuronal damage including synapse degeneration and axonal demyelination in HIV infection and other neurodegenerative diseases [264-267] In addition to the role of MMP-9 in the remodeling of the synapses during disease, part of MMP-9's damaging effects may be related to its ability to metabolize NGF [268] creating a favorable environment for a high ratio of proNGF to mature NGF. The partially protective role of NGF could also be explained by its effects on MMP-9. NGF decreased MMP-9 and TIMP-1 in the presence of HIV suggesting its role in suppressing MMP-9 toxicity. MMP-9 was also increased with proNGF stimulation alone but had no effect on TIMP-1 secretion from macrophages. This increase in MMP-9 secretion may explain why conditioned medium from proNGF stimulated macrophages in the absence of HIV is neurotoxic. Finally, studies of macrophage invasion have demonstrated a strong relationship between podosomes and MMP-9 secretion providing a link between the MDM phenotype and toxic activity [269,270]. The consistency of these observations makes MMP-9 a putative candidate for the neurotoxin. Indeed preliminary results indicate that MMP-9 induces a gradual accumulation of calcium in neurons and MMP inhibitors reduce toxicity. Much work is needed to understand how MMP-9 may be involved in neuropathogenesis, but it may serve as a potential biomarker or early indicator for disease prognosis.

Conclusion

These studies describe a new phenotypic characterization of neurotoxic macrophages that can be induced by HIV. This phenotype includes the formation of polarized podosomes, suppression of calcium spiking in macrophages and increased neurotoxin production that

correlated with the ability of conditioned medium from these cells to induce calcium dysregulation in neurons. Proteins showing the most robust changes in the medium in response to HIV included antiangiogenic factors and MMP-9. The formation of podosomes and neurotoxin production was dependent on neurotrophin $p75^{NTR}$ signaling and neurotoxicity was partially suppressed by TrkA signaling. Stimulation of macrophages with HIV increased $p75^{NTR}$ co-localization with both TrkA and sortilin providing the opportunity for receptor interactions. Co-stimulation of macrophages with proNGF resulted in an increased neurotoxic phenotype although many functions were not changed compared to HIV. This suggested that proNGF and HIV are producing the neurotoxic phenotypes through converging pathways. The most significant effects were the observations that co-stimulation with NGF and HIV suppressed the neurotoxic phenotype in macrophages by increasing calcium spiking, suppressing podosomes and reversing many of the protein secretory changes induced by HIV, including MMP-9. These differential effects of proNGF and NGF on HIV activation provides a potential novel therapeutic avenue for controlling macrophage phenotypes during HIV associated cognitive disorders and other inflammatory diseases.

CHAPTER 4: NEUROTROPHIN-CXCR4 INTERACTIONS REGULATE THE TOXIC ACTIVATION OF MACROPHAGES BY HIV

Introduction

Macrophages and microglia are the cellular targets that carry the major burden of HIV in the central nervous system. Activation of these mononuclear phagocytes by HIV has been correlated to disease severity in patients with HIV associated cognitive disorders[79]. Though mechanisms of macrophage activation by HIV are not fully understood, engagement of the surface protein on the viral envelope, gp120, with the chemokine receptors, CCR5 or CXCR4, on the host cell is thought to play a major role [86,102,271,272]. This gp120 induced macrophage activation is independent of infection and binding to the HIV primary receptor, CD4 [86,100,103,271,273,274]. CCR5 preferring viral strains are the main HIV variants in the brain although distinct brain variants preferring CXCR4 have been identified in cerebrospinal fluid (CSF)[48]. The binding of these virions is not exclusively restricted to their preferred receptors and CXCR4 preferring strains can activate and infect macrophages although with reduced efficiency relative to CCR5 preferring strains. Although most virus in the CNS is CCR5 preferring, studies have shown that both CXCR4 preferring and CCR5 preferring envelope proteins can cause neurotoxicity [275]. The specific role of viral interactions with each chemokine receptor has been extensively debated but interactions with CXCR4 have always been associated with neurotoxicity whereas CCR5 has been implicated in both toxicity and neuroprotection depending on the context. The exact contribution of each co-receptor to the development of neural damage remains controversial.

In the previous chapter we showed that hMDM stimulation with CCR5 preferring virions, HIV_{ADA}, produced a neurotoxic phenotype consisting of podosome formation and the secretion of neurotoxins. This toxic phenotype was similar to what was seen during hMDM stimulation with proNGF in Chapter 1, and HIV toxicity was exacerbated during co-stimulation with proNGF. However co-stimulation with mature NGF was able to reduce toxicity caused by HIV. This suggested a close relationship between neurotrophin and HIV signaling in the hMDMs. Interactions between neurotrophins and CXCR4 signaling have been suggested by studies of HIV infection and macrophage activation. NGF has been reported to suppress or enhance viral replication in hMDMs [171,174]. Samah et. al has shown that NGF can increase SDF induced migration in HIV infected and non-infected macrophages by increasing the cell surface expression of the SDF-1 receptor, CXCR4 [175,176]. However the nature of the interaction of NGF with CXCR4 expression or function has not been addressed.

NGF-CXCR4 interactions may be direct or indirect. The possibility of direct interactions was raised by data from Moses Chao's group showing that Trk receptors can be transactivated by G-protein coupled receptors [276-280]. In addition, transactivation of CXCR4 by the insulin-like growth factor-1 receptor has been reported in human breast cancer cells[281]. These studies and our previous observations raised the possibility that neurotrophin receptors and chemokine receptors may interact to control cytoskeletal dynamics and macrophage functions. In this study we show for the first time that CXCR4 forms complexes with the $p75^{NTR}$ and TrkA to modulate neurotoxin production during HIV exposure.

Materials and Methods

Isolation and culture of human monocyte-derived macrophages

Human buffy coat leukocytes were purchased and shipped within 24 hours after blood draw from healthy donors at the New York Blood Center (http://nybloodcenter.org/), a non-profit organization specific for the collection and distribution of blood for clinical and research purposes. All research use was screened by the center and no personal identifiers were sent with the shipment. Such use is exempt from review under NIH guidelines, exemption 4 of 46-101(b). Blood was diluted 1:1 with phosphate buffered saline (PBS) and was layered on top of Ficoll-Paque (GE Healthcare 17-1440-03). Blood/Ficoll-plaque was centrifuged at 500 X g for 25 min and the peripheral blood mononuclear cells (PBMCs) were collected from the PBS/Ficoll-Paque interface. PBMCs were washed in red blood cell lysis buffer (Sigma R7757) to remove any red blood cell contamination. PBMCs were centrifuged at 450 X g, the supernatant aspirated and the pellet re-suspended in Dulbecco's modified eagle medium (DMEM) with high glucose, 10% fetal bovine serum (Gibco 160000-044) and 20 μg/ml gentamicin (Gibco 15750-60). Cells were aliquoted into low adhesion 6 well plates (Corning 3471) at a density of approximately 10^7 cells/well. PBMCs were cultured for 5-7 days to allow monocyte attachment. Remaining white blood cells were washed, from the plate yielding a pure monocyte/macrophage culture. The adherent cells were differentiated into monocyte-derived macrophages (hMDM) using human GM-CSF (15 ng/ml) in complete DMEM for one week. Monocyte experiments were carried out within 1 hour of PBMC isolation to prevent cell attachment.

Primary cultures of rat forebrain

All animal work was done in accordance with NIH animal welfare guidelines and was approved by the University of North Carolina- Chapel Hill Institutional Animal Care and Use Committee board (approval number 14-147.0). Timed gestational embryonic day 9 (E9) pregnant female Long-Evans rats were delivered from Charles Rivers and maintained in UNC animal husbandry until the time of experiments. At gestational day E17, rats were sacrificed by anesthetizing with isoflurane drop method until breathing and the heart stopped. A thoracotomy was then performed prior to removal of the fetal material. For primary neuronal cultures, fetal material was rinsed in 70% ethanol and placed in HEPES-buffered Hank's balanced salt solution (HBSS) on ice for anesthesia before removal of the brain. The tissue was extensively washed and the cortex/hippocampus was dissected from each brain and cleaned of Dura-arachnoid membrane and visible vessels. The tissue was transferred to a 15 ml tube containing 5 ml calcium-magnesium free-HBSS + 2.4 U/ml dispase + 2 U/ml DNase I and incubated for 25-30 min at 36° C. Tissue was triturated and allowed to settle for 2 min. The suspended cells were transferred to a 50 ml culture tube containing 25 ml of minimum essential medium (MEM) with glutamine + 10% fetal bovine serum + 20 μg/ml gentamicin. After several rounds of trituration in 2-3 ml calcium-magnesium free HBSS, dissociated cells were seeded at a density 20,000 cells/cm^2 on poly-D-lysine-treated coverslips for imaging and staining or 50,000-100,000 cells/cm^2 in 60-100 mm plastic dishes for Western blots. After 24 hours, cultures were transferred to Neurobasal medium with B27 supplement. The resulting cultures were >95% neurons at day 4 after seeding.

HIV activation of hMDM

Differentiated hMDM grown on poly-D-lysine coated coverslips were transferred to DMEM containing 1% FBS and stimulated for 1 or 24 hours using four different conditions: HIV_{ADA}, HIV_{ADA} +NGF human recombinant protein (100 ng/mL, Sigma N1408), HIV_{ADA} +proNGF human recombinant protein targeted to high affinity sites (1 ng/ml, Alamone N-280), or vehicle. A replication incompetent stock of inactivated HIV_{ADA} was used for these studies. The concentration of each neurotrophin was selected to maximize the effects of NGF on the macrophages while also maximizing the difference between NGF and proNGF. In inhibition studies the following inhibitors were used: AMD3100 (CXCR4), CCR5 neutralizing antibody or Maraviric (CCR5) and GW441756 (TrkA).

Immunostaining

The cells were gently washed and fixed in 2% paraformaldehyde in PBS. Cells were washed 3X in PBS and incubated in 3% normal goat serum for one hour. Incubation of primary antibodies was carried out overnight at 4^{o} C. Cells were stained using antibodies to: $p75^{NTR}$ (Millipore cat #07-476, 1:500), TrkA (Santa Cruz cat #SC-80961, 1:500), pCXCR4 (ABCAM ab74012) or CXCR4 (R+D System cat# MAB170). Cells were washed in PBS and incubated with species specific secondary antibodies conjugated to Alexa 488, 568 or 594 (Molecular Probes) in the dark for 1 hour at room temperature. F-actin was stained using Alexa488 phalloidin (1:50, Molecular Probes) to show structural changes. Coverslips were then mounted using Fluoromount (Southern Biotech 0100-01) and digitally imaged on an Olympus XI71 microscope.

Analysis of stain intensity for individual cells or regions of interest within the cells was accomplished using Metamorph software. Co-localization of pCXCR4 foci with TrkA or $p75^{NTR}$ staining was analyzed by thresholding the regions of interest and using the co-localization tool in MetaMorph software to quantify the extent of overlap.

Co-immunoprecipitation

Human monocyte-derived macrophages were stimulated with HIV, NGF+HIV or proNGF+HIV for 10 min in serum free DMEM which was previously determined to be the peak time for identification of protein-protein interactions. Cells from two 60 mm or one 100 mm dish were harvested using 1.5 ml lysis buffer (1X RIPA buffer (Thermo Scientific 89900), 1:100 phenylmethylsulfonyl fluoride (PMSF, Pierce Chemicals PI 36978), 1:100 Halt® protease inhibitor cocktail (Thermo scientific cat# 1861228). Protein concentration was measured by BCA assay (Thermo Scientific 23225). 50 µl of ProteinA/G Plus-Agarose beads (Santa Cruz Cat#sc-2003) and 10 µg of CXCR4 antibody (Alamone cat#ACR-014 was added to 1ml of lysate and incubated overnight. Excess antibody was washed three times in RIPA buffer and beads containing immunoprecipitated protein were boiled in Laemelli sample buffer to isolate protein from beads. The Isolated proteins were resolved on SDS-Page gels as discussed below.

Western blots

Human monocyte-derived macrophages were stimulated with HIV_{ADA}, HIV_{ADA} +NGF or HIV_{ADA} +proNGF for time periods of 10 min. The hMDMs were pretreated with the specific inhibitor for 30 minutes prior to stimulation. At the indicated time, cells from

two 60 mm or one 100 mm dish were harvested using 1.5 ml lysis buffer (1X Ripa buffer (Thermo Scientific 89900), 1:100 phenylmethylsulfonyl fluoride (PMSF, Pierce Chemicals PI 36978), 1:100 Halt® protease inhibitor cocktail (Thermo scientific cat# 1861228). Protein concentration was measured by BCA assay (Thermo Scientific 23225). Protein lysate was resolved on SDS-Page gels (Biorad cat# 456-1034) and transferred to a nitrocellulose membrane (Biorad 162-0112). The membranes were blocked in 2% milk plus 0.01%Tween for one hour at room temperature. The primary antibodies were incubated overnight at four degrees. The membranes were then washed in PBS+1% Tween and incubated in secondary antibody for one hour at room temperature. The membranes were then washed again and imaged using the Odyssey or film imaging system. The primary antibodies used were 1:500 $p75^{NTR}$ (Millipore 05-446), 1:500 TrkA (Millipore 06-574), 1:500 pCXCR4 (ABCAM ab74012), 1:500 CXCR4 (R+D System cat# MAB170), 1:500 GRK2 (ABCAM cat#AB113643). Secondary antibodies were hoseradish peroxidase (HRP) conjugated secondary antibodies (Goat anti-Rabbit IgG, cat# AP132P and Goat anti Mouse IgG cat# AP124P) and SuperSignal West Pico detection (Thermo Scientific, #34080).

Flow Cytometry

PBMCs and hMDMs grown in ultra-low adhesion 6-well plates were stimulated with NGF or proNGF in 1% DMEM for 1 hour (PBMCs) or overnight (hMDM). Cells were removed from the low adhesion wells and centrifuged for 5 minutes at 450 x g. Cellular pellets were re-suspended and fixed in a Fluorfix solution (Biolegend 420801) for 20 minutes at room temperature. Fixed cells were then treated with permeabilization buffer (EBioscience 020-8333-56) and centrifuged for five minutes at 450 x g at 4° C. The cell pellet was re-

suspended in permeabilization buffer and centrifuged at 450 X g at 4° C. Cells were re-suspended in 100 µl of permeabilization buffer plus antibody (1.5 µl $p75^{NTR}$ Alamone Labs ANT-007-F ; 20µl; TrkA R&D Systems FAB1715P; 5µl CD 206 Biolegend 321114; 5µl CD16 Biolegend 302008 and 5µl CD 163 Biolegend 333607) at room temperature for twenty minutes. The stained cells were washed three times in cell staining buffer (Biolegend 420201). Flow cytometry was performed on a FACS Calibur (Becton Dickinson, San Jose, CA) using direct immunofluorescence with at least 100,000 events. Monocytes were determined by populations of cells with high forward scatter and low side scatter. All cells were gated to remove debris. Three color staining analyses were utilized. Cells were analyzed according to side scatter and receptor bound fluorescence, and data was collected with logarithmic amplifiers. Fluorescence spillover compensation was estimated using single-stained and unstained samples with the Cell Quest software (BD). After collection, data was further analyzed with FlowJo software (TreeStar Inc., Ashland, OR).

Neurotoxicity of macrophage-conditioned medium

Pilot studies indicated that a 30 minutes stimulation of hMDM in serum free DMEM was sufficient to induce an MDM phenotype which persisted for several hours after removal of the stimulus. Thus, the cells could be primed with neurotrophin in serum free DMEM for 30 minutes, washed and replaced with serum free medium containing HIV_{ADA} virions. The resulting macrophage-conditioned medium (MCM) was collected and centrifuged at 2500 rpm for 10 minutes to remove any cells and then frozen in aliquots at -80° C. The neurotoxic activity of the medium was tested on primary rat neurons cultured on coverslips. Neurons at 6-12 days in vitro were loaded with the calcium indicator, Fluo-4 AM (2 µM, Molecular

Probes, Inc., Eugene, OR) in aCSF (aCSF: NaCl 137 mM, KCl 5.0 mM, $CaCl_2$ 2.3 mM, $MgCl_2$ 1.3 mM, glucose 20 mM). After 30 minutes, the coverslip was transferred to a specialized stage for imaging. Cells were maintained in aCSF and time lapse digital images were captured automatically by the Metamorph® system. Images were captured every 6 seconds for 6 minutes to assess acute effects and every min for 60 minutes to assess delayed effects. Three pre-stimulation measurements were taken to establish basal levels of fluorescence at the beginning of each experiment. Neurons were stimulated with MCM at a 1:5 dilution. The increase in fluorescence intensity within each cell was then measured relative to the baseline fluorescence to correct for cell to cell differences in dye loading and intrinsic fluorescence. For most studies, cellular responses were averaged across all cells from at least triplicate runs to provide an indication of the "typical" response. In some cases individual cell response patterns are shown where the average masked important cell-specific profiles.

Measurement of macrophage calcium responses

Macrophages cultured on coverslips were pretreated with the specific inhibitor and the calcium indicator, Fluo-4 AM in aCSF for 30 minutes. Three time lapse digital images were captured followed by stimulation with $HIV_{ADA.}$ Changes in fluorescence intensity within each cell were measured every 6 sec for 20 min and analyzed as described above. Peaks were assessed by calculating sequential changes in fluorescence intensity and identifying increases that were greater than 2 standard deviations above the average baseline (>20 fluorescence units).

Statistical analyses

Graphpad Prism software was used for data summaries and graphics. Parametric statistics were used to evaluate most changes induced by NGF or proNGF relative to matched or unmatched control samples depending on the experiment. In cases where the data were not normally distributed non-parametric statistics were used. Means and standard errors were calculated for at least three replicate experiments. T-tests were used for paired comparisons, analysis of variance with repeated measures for temporal data and Chi-square for the analysis of cell populations. The statistical values in the text represent results from t-tests unless otherwise indicated. A probability of <0.05 for rejection of the null hypothesis was considered significant unless otherwise indicated.

Results

CXCR4 but not CCR5 is necessary for neurotoxin production

To understand co-receptor involvement in neurotoxicity, hMDMs were pretreated with a CCR5 neutralizing antibody or CXCR4 specific inhibitor , AMD3100, for 30 minutes, washed and then exposed to HIV_{ADA} for 1hour. Conditioned medium was collected and placed onto rat cortical neurons preloaded with the calcium indicator dye, Fluo-4. Macrophage conditioned medium (MCM) collected after HIV_{ADA} treatment induced a rapid increase in calcium that recovered partially during the acute phase followed by a prolonged rise in delayed calcium and subsequent formation of varicosities. Vehicle (aCSF), virus alone or medium from unchallenged macrophages produced negligible or modest (unchallenged MCM) calcium increases and no neuronal damage was seen. Blocking CCR5 signaling before HIV_{ADA} exposure slightly increased delayed calcium dysregulation while conditioned medium from macrophages with the CXCR4 antagonist had significantly reduced neurotoxin production (Fig.3.1). This indicated that CXCR4 is the principle co-receptor necessary for release of neurotoxins, even when stimulated with CCR5-preferring virions.

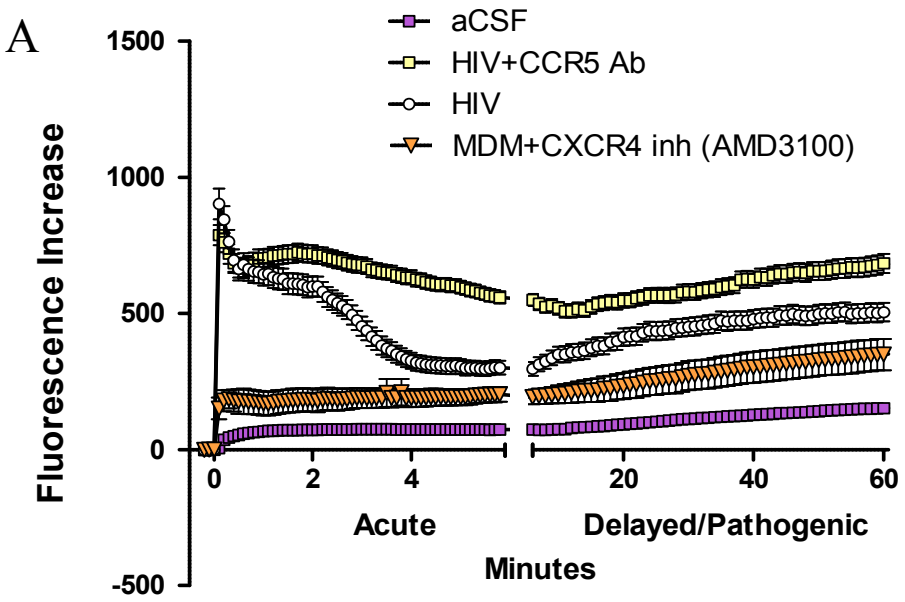


Figure 3.1 CXCR4 but not CCR5 is necessary for neurotoxicity. A. Human monocyte derived macrophages were pretreated with the CXCR4 specific inhibitor AMD3100 or a CCR5 neutralizing antibody before exposure to HIV_{ADA} for 1 hour. Conditioned medium was collected and placed onto rat cortical neurons and calcium dystabilization was assessed in two phases. HIV (white circle) induced a rapid rise in calcium acutely followed by a plateau. A gradual and prolonged increase in calcium was seen during the delayed phase. Blocking CCR5 (yellow square) slightly increased calcium dysregulation during both the acute and delayed phase while inhibition of CXCR4 signaling (orange triangle) reduced calcium dysregulation.

CXCR4 regulates podosome and ruffle formation in hMDMs

In the previous chapter, we showed that conditioned medium from cells that possess more actin rich ruffled structures were less toxic to neurons while medium from cells with podosomes were correlated with greater neurotoxin production. In addition, exposure to HIV_{ADA} induced the formation of the podosome bearing phenotype in a $p75^{NTR}$-dependent manner. To assess the role of the HIV co-receptors, CXCR4 or CCR5, in toxin formation we inhibited CXCR4 signaling with AMD3100 and CCR5 signaling with maraviroc during exposure to HIV_{ADA} virions for 1 hour. As previously shown, the proportion of cells expressing podosomes was increased in response to HIV. Inhibition of CCR5 signaling during HIV_{ADA} exposure had no significant effect on the expression of ruffles (Fig. 3.2A) or podosomes (Fig 3.2B) compared to HIV treated cells. In contrast, in the absence of CXCR4 signaling, a large increase in the proportion of ruffled cells was seen ($p=0.008$ vs. HIV). There was no significant difference in the total number of podosome bearing cells; however, the density of podosomes within cells was significantly reduced compared to HIV treated cells. Low numbers of podosomes were often seen diffusely scattered through the cell and fewer cells had distinct clusters of podosomes ($p=0.012$, AMD3100+HIV vs. HIV, not shown).

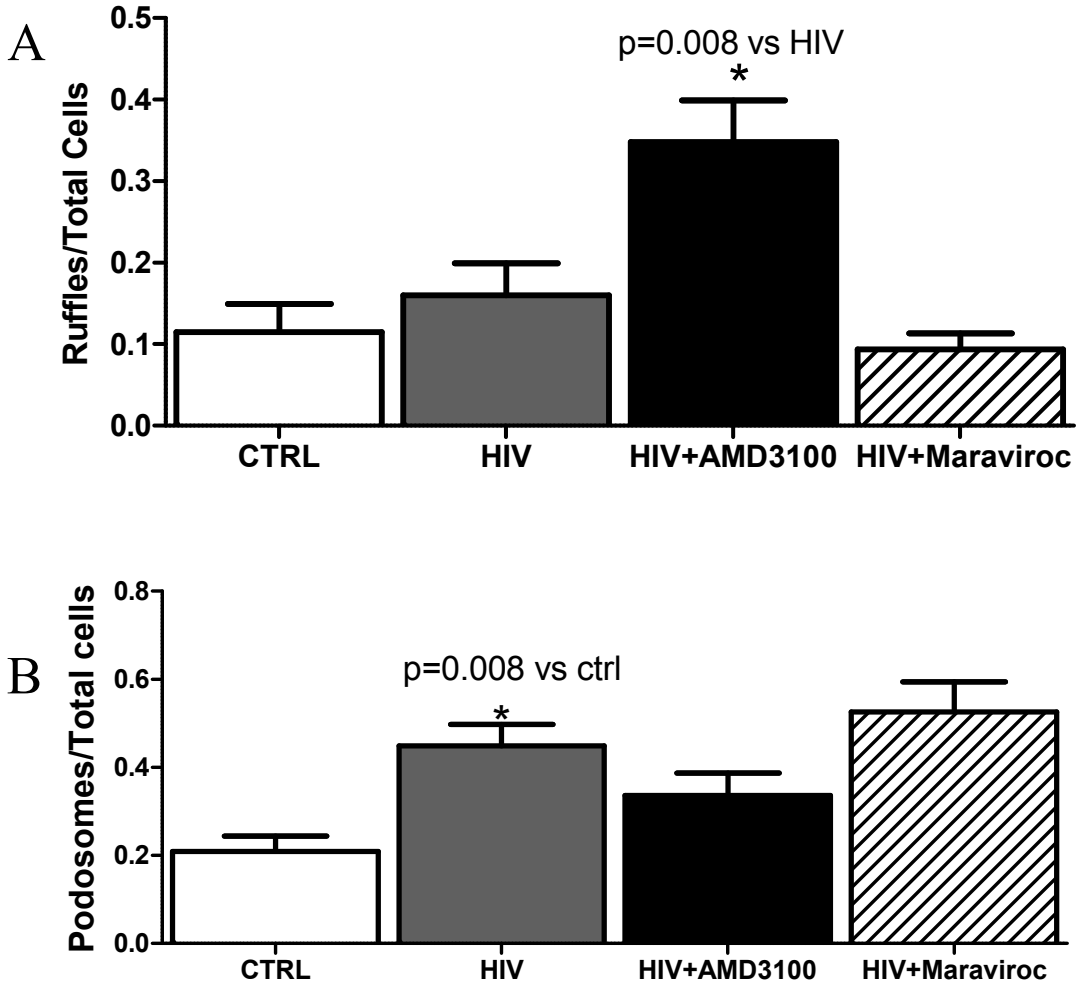


Figure 3.2 CXCR4 but not CCR5 suppressed ruffles in hMDMs. A. HIV had no effect on cells bearing actin rich ruffled structures in hMDMs. Suppression of CXCR4 signaling with specific inhibitor AMD3100 increases the number of ruffled bearing cells in culture. Suppression of CCR5 signaling with the specific inhibitor maraviroc had no effect on ruffled structures. B. HIV increased the number of hMDM with podosomes. Suppression of CCR5 and CXCR4 signaling had no effect on the proportion of cells possessing podosomes (n=10).

CXCR4 neutralizing antibody blocks intrinsic and NGF-induced Calcium Spiking

Blockade of CXCR4 with a neutralizing antibody prior to the addition of HIV or HIV in combination with NGF or proNGF strongly suppressed calcium spikes across all conditions (Fig 3.3;$p<0.001$, 2 way ANOVA). Calcium spiking in normal cells in the absence of any treatments was blocked by 81.1% in the presence of the CXCR4 antibody ($p<0.05$), and spiking induced by NGF in the presence of HIV was blocked by 98.6% indicating that CXCR4 activation may be necessary for the NGF-induced increase in calcium signaling. To test this possibility, CXCR4 was blocked and the hMDM stimulated with NGF in the absence of HIV. The CXCR4 block significantly suppressed the spike frequency by 61.4% indicating that most of the calcium channel activity was CXCR4 dependent.

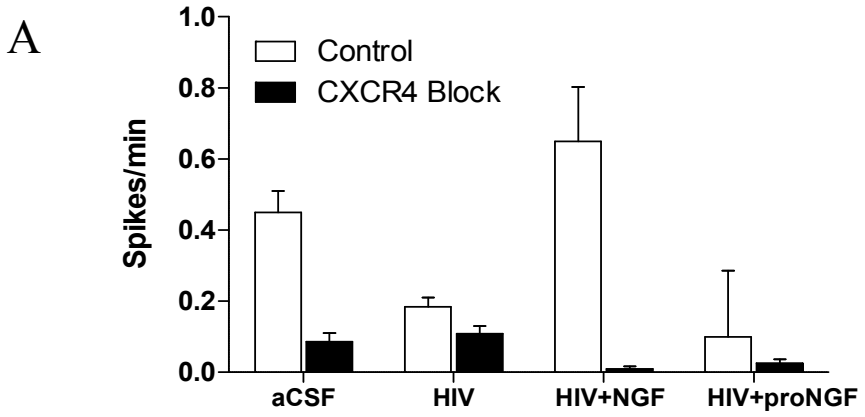


Figure 3.3 CXCR4 signaling is necessary for calcium spiking. HIV and proNGF+HIV normally suppressed calcium spiking in hMDMs whereas co stimulation of NGF with HIV increased calcium spiking. In each case calcium spikes were strongly suppressed by inhibition of CXCR4 (n=10, controls; n=3 cxcr4 block).

NGF increases CXCR4 phosphorylation

The activation of CXCR4 with its natural ligand stromal derived factor-1 (SDF-1) is dependent on phosphorylation, however it is unclear if HIV causes phosphorylation of CXCR4. To determine if HIV and neurotrophins influence CXCR4 phosphorylation, we exposed hMDMs to HIV_{ADA} for 10 minutes and evaluated CXCR4 phosphorylation by western blotting. A 10 minute exposure to HIV_{ADA} slightly increased phosphorylation of CXCR4 by 13$\pm$0.5% compared to untreated hMDMs (Fig3.4; p=0.03). Co-stimulation of hMDM with proNGF + HIV_{ADA} had no effect on HIVs ability to phosphorylate CXCR4. In contrast, HIV induced CXCR4 phosphorylation was increased by co-stimulation with NGF (+13.27%; p=0.02 vs HIV_{ADA}). The role of NGF was supported by demonstrating that CXCR4 phosphorylation was reduced by 43.75% in the presence of the TrkA inhibitor, GW 441756 (p=0.005 vs NGF+HIV_{ADA}).

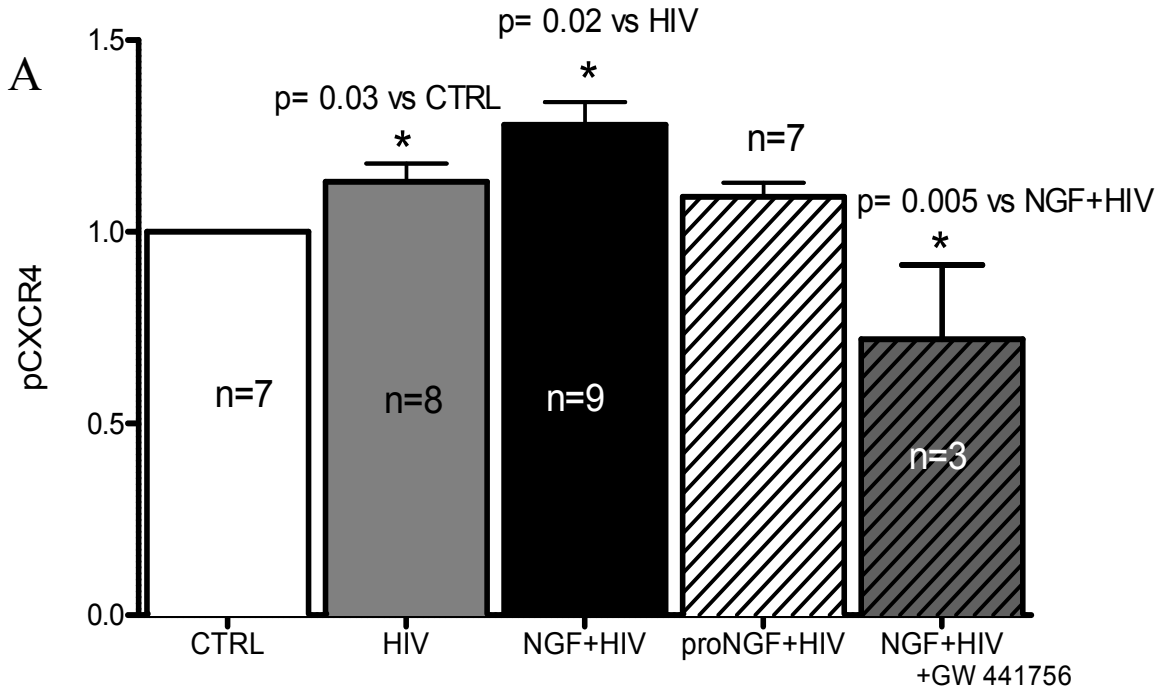


Figure 3.4 NGF induced phosphorylation of CXCR4 in response to HIV. A. HIV slightly yet significantly increased phosphorylation of CXCR4. Co-stimulation with NGF further increased HIV induced phosphorylation of CXCR4. TrkA inhibition with the specific inhibitor GW441756 reversed the phosphorylation of CXCR4 induced by NGF + HIV.

CXCR4 is expressed in same regions as TrkA and p75 ^{NTR}

To understand how NGF may regulate CXCR4 signaling, we assessed CXCR4 localization in hMDMs. Total CXCR4 was diffusely expressed in hMDM while phosphorylated CXCR4 (pCXCR4) was expressed in more sequestered regions in actin rich regions where neurotrophin receptors are expressed as illustrated in Chapter 1(Fig 3.5B and C). To assess the potential overlap of pCXCR4 with either p75 ^{NTR} or TrkA in double stained cells, regions of interest were identified, thresholded for stained objects and quantified by Metamorph® software. PCXCR4 overlapped with TrkA staining by 68.4$\pm$0.3% (Fig 3.5B and D) and p75 ^{NTR} by 64.3+0.5% (Fig.3.5c and E). HIV_{ADA} exposure reduced pCXCR4/TrkA and pCXCR4/p75^{NTR} overlap to 14.3% and 35.8%, respectively ($p<0.001$, pCXCR4/TrkA and $p=0.001$, pCXCR4/p75 ^{NTR}) (Figs 3.5D and E). Co-stimulation with proNGF+HIV_{ADA} further decreased pCXCR4/p75^{NTR} overlap relative to HIV_{ADA} alone (-54.78%; $p=0.02$) (Fig 3.5E) but had no effect on pCXCR4/TrkA overlap. In contrast, co-stimulation with NGF+HIV_{ADA} resulted in a recovery of pCXCR4/p75 ^{NTR} overlap compared to HIV_{ADA} (35.76+0.8% HIV_{ADA} to 57.89+0.5% NGF+HIV_{ADA}; $p=0.05$) (Fig 3.5D) and partially recovered pCXCR4/TrkA overlap (14.26+0.3% HIV_{ADA} to 30.66+0.5% NGF+HIV; $p=0.008$) (Fig 3.5E). These data suggest that activated pCXCR4 is expressed in overlapping domains with neurotrophin receptors, p75^{NTR} and TrkA. This overlap is downregulated by HIV_{ADA} and can be reversed or exaggerated by mature NGF or proNGF signaling.

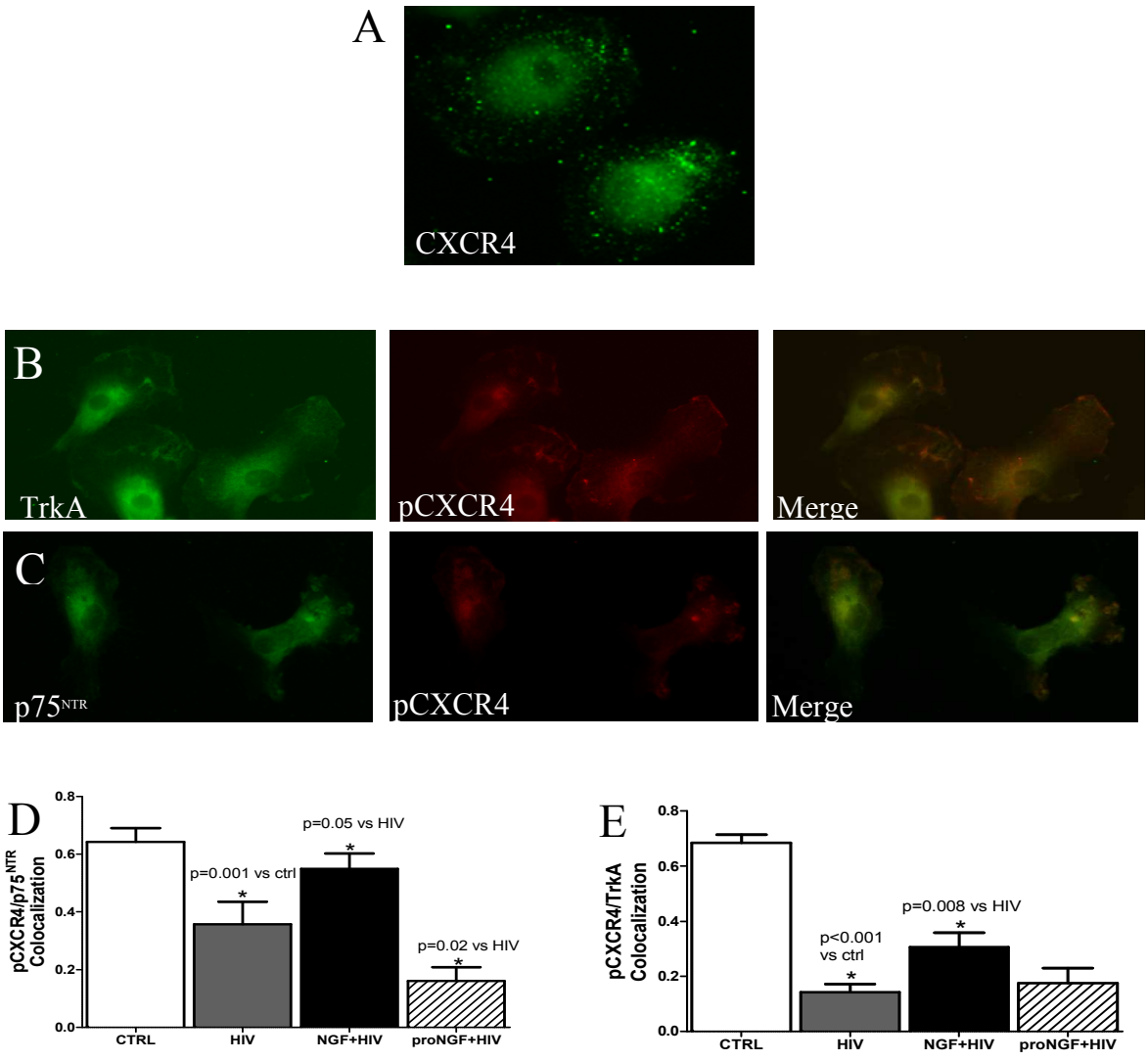


Fig 3.5 CXCR4 interacts with neurotrophin receptors. A. Total CXCR4 (green) is diffusely expressed in human macrophages. B. The p75 ^{NTR} (green) and pCXCR4 (red) were located in the same domains on human macrophages (co-localized, arrow). C. TrkA (green) and pCXCR4 (red) was located in the same domains on human macrophages (co-localized, arrow). D. Quantification of pCXCR4 overlaps with p75^{NTR}. HIV decreases this overlap. NGF co-stimulation with HIV restores pCXCR4/p75 ^{NTR} overlap while proNGF co-stimulation further decreases the overlap of these receptors(n=4). E. Quantification of pCXCR4 overlaps with TrkA. All treatments decreased this overlap. NGF co-stimulation with HIV partially restored pCXCR4/p75 ^{NTR} overlap while proNGF co-stimulation had no effect (n=4).

CXCR4 forms complexes with neurotrophin receptors

To confirm the interaction between CXCR4 and neurtrophin receptors, we immunoprecipitated total CXCR4 from hMDM lysates and probed for $p75^{NTR}$ and TrkA. Total CXCR4 co-immunoprecipitated with both a 75 kDa band recognized by a $p75^{NTR}$ antibody(Figs 3.6A and B) and a weak 140 kDa band recognized by an anti-TrkA antibody. However, for TrkA a stronger band at approximately 280 kDa was also seen which may represent a TrkA dimer as identified in other studies [282,283]. Stimulation of hMDMs with HIV_{ADA} for 10 minutes had no effect on co-immunoprecipitation of CXCR4 with $p75^{NTR}$ or TrkA.

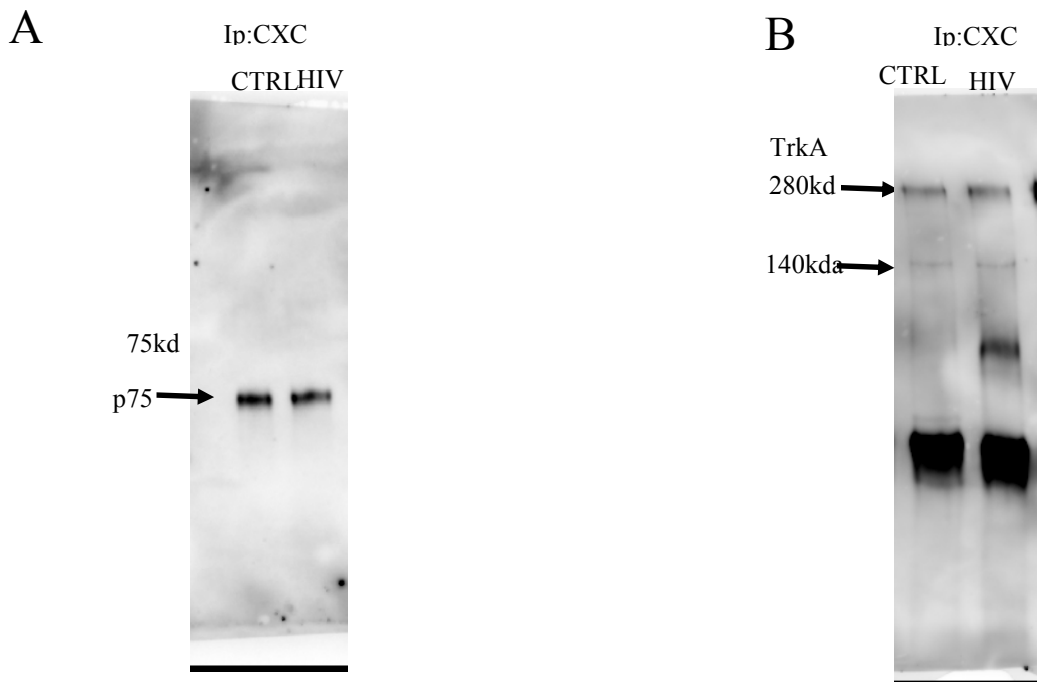


Figure 3.6 CXCR4 forms complexes with neurotrophin receptors p75 ^{NTR} and TrkA. A. A representative blot of p75^{NTR} (located at 75kDa) co-immunoprecipitated with CXCR4. HIV had no effect on p75 ^{NTR} /CXCR4 complexes. B. A representative blot of TrkA co-immunoprecipitated with CXCR4. A faint band was seen at 140kDa. A stronger band was seen at 280kDa representing a possible TrkA dimer. HIV had no effect on TrkA/CXCR4 complexes.

NGF increased GRK2/CXCR4 interactions in macrophages

The G-protein receptor kinase (GRK) family, and particularly GRK2, is thought to be responsible for phosphorylation of CXCR4 [284]. To assess the potential role of GRK2 interactions with CXCR4 during HIV and neurotrophin signaling, hMDMs were stimulated with HIV for 10 minutes in the presence or absence of proNGF or NGF. CXCR4 was immunoprecipitated from hMDM lysates and GRK2 was probed using western blotting techniques. CXCR4 co-immunoprecipitated with GRK2, however, HIV decreased CXCR4 interactions with the GRK2 band by 21% compared to untreated cells ($p=0.02$)(Fig 3.7A and B).Co stimulation of hMDM with proNGF + HIV had no effect relative to HIV alone, however, co-stimulation with NGF + HIV increased the interaction between GRK2 and CXCR4 by 46.8% compared to HIV($p,<0.01$). These data indicate that NGF stimulation encourages an interaction between GRK2 and CXCR4 that is down regulated during HIV exposure and may explain the NGF mediated increase in phosphorylation of CXCR4.

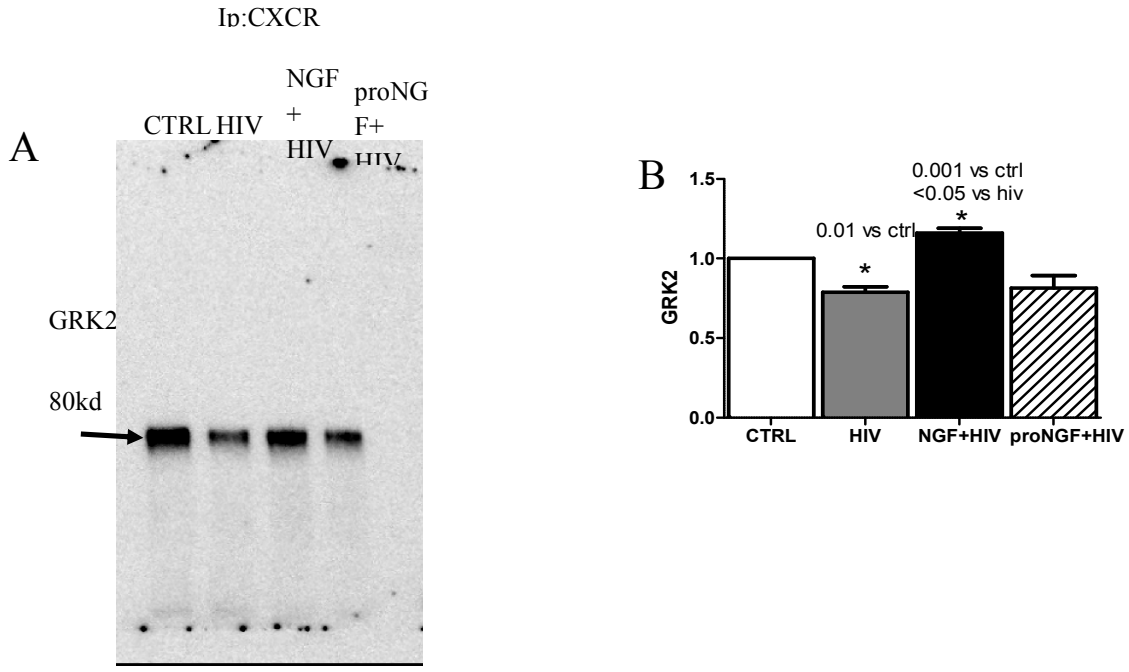


Figure 3.7 NGF encourages GRK2 and CXCR4 complex formation. A. A representative blot of GRK2 (located at 80kda) co-immunoprecipitated with CXCR4. B. HIV reduces GRK/CXCR4 complexes. NGF co-stimulation increases GRK2/CXCR4 complexes during HIV signaling. ProNGF has no effect on the ability for GRK2 and CXCR4 to form complexes (n=4).

Discussion

In our studies we have shown that both HIV and the neurotrophins encourage the development of unique hMDM phenotypes best characterized by the expression of ruffles (induced by NGF) or podosomes (induced by HIV or proNGF). The activation of toxin production in the macrophages was closely related to the presence of podosomes and inversely related to ruffles and calcium spikes. In this study we have shown that the secretion of neurotoxins in response to HIV is dependent on CXCR4 but not CCR5 signaling. This was a surprising result because the HIV variant used in these studies is a CCR5 preferring strain. However, studies showing co-receptor preference have been based on infection data and may have less relevance to CD4-independent interactions of the virus with the MDM. Indeed, even with infection as the endpoint, studies have shown overlap in the ability of virions to use each receptor. The effects of HIV virions appear to be closely tied to structural changes in the MDM. Evidence from our studies and the studies of others that HIV encourages podosome remodeling [285] indicate that the podosome bearing phenotype is more neurotoxic. This is supported by the observation that cells possessing actin rich ruffles produced medium that was much less neurotoxic. CXCR4's involvement in suppressing ruffled bearing cells and encouraging a denser population of podosomes in macrophages further suggest that CXCR4 is the co-receptor necessary for neurotoxicity as blockade of CCR5 had no effect on actin structures.

Inhibition of CXCR4 signaling resulted in many phenotypes that were seen with NGF stimulation of macrophages in Chapter 1. The Gras group laid the foundation for an interaction between NGF signaling and CXCR4 by showing that NGF increased the migration of monocytes in the presence of CXCR4's natural ligand SDF-1. The effect was

independent of the absence [176] or presence of HIV [175]. Samah et.al, (2009) also showed that NGF increased the surface expression of CXCR4. However, they did not explain how NGF may be regulating CXCR4 activity. In this study we show that HIV increased phosphorylation of CXCR4, albeit at low levels. This HIV induced phosphorylation was increased by NGF co-stimulation but was not changed during proNGF co-stimulation. Activated pCXCR4 was specifically found in overlapping domains with NGF receptors $p75^{NTR}$ and TrkA at the outer edges of the hMDMs. Also, HIV decreased the overlap of pCXCR4 and neurotrophin receptors. The ability of NGF to restore the overlap suggested that these interactions may suppress neurotoxin secretion during HIV exposure. ProNGF co-stimulation with HIV exacerbated HIV_{ADA}'s ability to decrease pCXCR4/p75 overlap which could explain the increased secretion of toxins from hMDMs treated with HIV and proNGF. Complexes formed between total CXCR4 and neurotrophin receptors $p75^{NTR}$ and TrkA confirmed an interaction between the two classes of receptors. In this case HIV did not affect the ability of CXCR4 to form complexes with p75 and TrkA. However these experiments did not address specific interactions with pCXCR4 complexes which may represent a fraction of the total signal.

A possible mode of CXCR4 phosphorylation may be through GRK2. The G protein kinase (GRK) family has been implicated in phosphorylation of CXCR4 although the exact family member has not been identified. Overexpression of GRK2 has been shown to increase SDF-1 mediated internalization of CXCR4, the step known to follow phosphorylation of this receptor. However, GRK2 has been suggested to downregulate CXC4 signaling[284], leaving doubt regarding the possible functional role of GRK2 mediated phosphorylation of CXCR4. Currently there are no good inhibitors of individual members of this family to

determine which, if any, GRK member is involved in the phosphorylation of CXCR4. Complexes of CXCR4 with GRK2 were reduced by HIV while co-stimulation of NGF and HIV encouraged CXCR4/GRK2 complexes. This increased interaction of GRK2 and CXCR4 with NGF stimulation may explain the NGF induced phosphorylation of CXCR4, but more studies are needed to confirm the potential role of GRK2 in CXCR4 signaling.

Conclusion.

In these studies we have shown that interactions of HIV with the HIV co-receptor CXCR4 are necessary for the formation of HIV induced neurotoxic phenotypes seen in macrophages. A direct interaction of neurotrophin receptors with CXCR4 was found indicating that neurotrophin and chemokine signaling are intimately related. NGF-dependent calcium spikes are substantially blocked by CXCR4 antagonists and NGF stimulation facilitated co-localization of CXCR4 with the neurotrophin receptors and interactions with GRK2, effects that may encourage phosphorylation since blockade of the $p75^{NTR}$ suppressed the phosphorylation of CXCR4. Thus, $p75^{NTR}$/CXCR4 interactions lead to phosphorylation and toxicity whereas TrkA/CXCR4 interactions reduce toxicity. Importantly, the ability of NGF and CXCR4 antagonists to suppress toxicity suggests an important avenue for the development of therapies that control toxic activity.

DISCUSSION

Summary of findings

In this study we have identified a neurotoxic phenotype in human monocyte derived macrophages that is stimulated by HIV interactions with CXCR4 and regulated by neurotrophins. We have confirmed that macrophages and monocytes express $p75^{NTR}$ and TrkA in high abundance and respond to their natural ligands proNGF and NGF, respectively. Macrophages also express another known $p75^{NTR}$ signaling partner, sortilin. A direct interaction of CXCR4 with both neurotrophin receptors was confirmed by immunocytochemistry and co-immunoprecipitation techniques. The $p75^{NTR}$, TrkA, sortilin and phosphorylated CXCR4 were all found in focal regions on the outer extremities of macrophages in overlapping domains. This expression provided an optimal environment for signaling interactions between HIV and the neurotrophins, proNGF and NGF.

Medium from HIV stimulated macrophages caused calcium dysregulation and cytoskeletal damage in neurons. Key macrophage phenotypes involved in neurotoxin production were the formation of podosomes and suppression of calcium spiking. In contrast, macrophages that expressed actin rich ruffled structures and increased calcium spiking were associated with less neurotoxin production. Treatment with proNGF enhanced the effects of HIV producing a more neurotoxic phenotype and increasing podosome formation in a $p75^{NTR}$ and CXCR4 dependent manner. The podosomes were often clustered in one quadrant of the cell and correlated with an increase in migration across a matrigel barrier. CXCR4 mediated calcium spiking was also reduced with both proNGF and HIV. HIV induced neurotoxin production was partially suppressed by inhibition of $p75^{NTR}$ and increased by blocking TrkA signaling. Proteomic analysis of conditioned medium showed that proNGF and HIV induced a similar profile by increasing anti-angiogenic factors and MMP-9 secretion. Proteins that antagonize

the effects of these factors such as TIMP-1 were reduced. These effects were not closely related to the secretion of conventional inflammatory cytokines. Taken together, these data indicate that proNGF and HIV produce similar neurotoxic phenotypes in macrophages by signaling through converging pathways that synergize to produce a more neurotoxic inflammatory phenotype.

NGF signaling produced an opposing phenotype in the macrophages. NGF under normal conditions increased actin rich ruffled cells and calcium spiking which correlated with a non-neurotoxic phenotype. NGF ruffle formation was mediated through TrkA. When co-stimulated with HIV, NGF reversed the effects of HIV on calcium spiking and podosome polarization. While neurotoxin production was suppressed with NGF stimulation alone, co-stimulation of NGF+HIV partially suppressed neurotoxicity. In the proteomic analysis of medium from macrophages treated with NGF+HIV the proteins induced by HIV were normalized to control levels including many growth factors with unknown functions. To determine the basis for the differential interactions between HIV and the neurotrophins, we explored signaling interactions with the HIV chemokine co-receptors. We identified CXCR4 as the co-receptor necessary for the neurotoxic phenotype induced by HIV. A small increase in CXCR4 phosphorylation was seen when macrophages were stimulated with HIV. This was significantly increased with the addition of NGF in a TrkA dependent fashion. ProNGF had no effect on phosphorylation suggesting that phosphorylation of the receptor may be involved in suppressing the neurotoxic phenotype. The possibility that phosphorylation of CXCR4 may be mediated by direct interactions between neurotrophin receptors $p75^{NTR}$ and TrkA and CXCR4 was confirmed for the first time by co-immunoprecipitation and co-localization in the same cell domains. CXCR4 also co-immunoprecipitated the G- protein

receptor kinase GRK2, an interaction that was reduced by HIV and enhanced by NGF. Together, these data have identified unique interactions of the neurotrophin receptors $p75^{NTR}$ and TrkA with HIV induced signaling through CXCR4. ProNGF and NGF exert opposing effects on the macrophages which have unique phenotypes and may offer new avenues for therapeutic intervention in HIV neuropathogenesis and other neurodegenerative diseases.

Discussion

Though the presence of activated macrophages in the brain has been a key correlate of the severity of HIV associated cognitive dysfunction, there are still many unanswered questions. The mechanisms that underlie both MDM activation and neuronal pathology are still poorly understood and are significant hurdles for the development of interventions that reduce CNS damage. Groups have reported that macrophages can be activated by HIV proteins independent of infection and binding to the primary receptor, CD4 [86,271]. This activation is thought to be through the engagement of the HIV surface protein, gp120, with the co-receptors CCR5 and/or CXCR4 on the host cell. Macrophage activation by HIV has been linked to the release of unknown toxins in the brain which lead to neuronal damage [18,286]. This process has provided a pivotal theoretical base for many different studies of HIV neuropathogenesis yet the identity of the toxins and their specific actions on neurons are still largely unknown. In spite of enormous effort over the past two decades no effective therapies have been discovered. To improve current therapeutic options we must have a better understanding of macrophage activation and neurotoxin production.

Macrophages are classically thought to be inflammatory cells widely linked to tissue destruction. However, it is now well accepted that macrophages are dynamic cells that respond to a wide range of cues driving diverse phenotypes ranging from anti-inflammatory, pro-inflammatory to reparative functions. This has led researchers to characterize the macrophage activation states into two groups: classical activation or M1 representing inflammatory phenotypes and alternate activation or M2 possessing anti-inflammatory qualities. As the anti-inflammatory functions became more diverse the category was further sub-divided into M2a induced by IL-4 and IL-13, M2b induced by immune complexes in

combination with IL-1b or LPS and M2c induced by IL-10, TGF-b, or glucocorticoids [23,31,287]. However, detailed studies of macrophage functions and phenotypes in response to different challenges, including results from this thesis, have shown that these cells can simultaneously express markers from different groupings. This is exemplified in HIV infections where some studies have classified macrophages driving M1 like phenotypes after HIV-Nef stimulation of M2 like cells [31], while others have suggested that macrophages take on both activation states depending on the stage of HIV replication [287]. This ambiguity in macrophage activation solidifies the complexity of macrophage activation and the need for a more detailed understanding of macrophage phenotypes outside of this classification system.

In spite of the large repertoire of macrophage functions, efforts to control their activation have generally been very crude. This is evident in the widespread use of steroidal and non-steroidal anti-inflammatory compounds as well as compounds with ill-defined actions such as minocycline. In this thesis a novel role for neurotrophin signaling has been identified which regulates inflammation in response to HIV. Currently there is very little data regarding neurotrophin signaling in macrophages and microglia despite their abundance in the central nervous system. In addition, over the past few years, it has become increasingly clear that the expression of the $p75^{NTR}$, in particular, is upregulated under pathological conditions. The reason for the upregulation and the function of these receptors under pathological conditions is largely unknown. Not only is receptor expression changing but studies have also shown that an imbalance in pro-neurotrophin versus mature neurotrophin levels is present in aging and neurodegenerative diseases such as Alzheimer disease [249]. Considerable effort has been expended to understand the significance of these changes for

neuronal function. Findings from this thesis show that macrophages and microglia are also primary targets of the neurotrophins and will need to be factored into studies of the role of neurotrophins in CNS function. Understanding how these neurotrophic factors may regulate macrophage functions is an important issue in the field and a potential novel avenue for therapeutic development.

HIV Neurotoxicity

The secretion of unknown neurotoxins from activated macrophages is necessary for neurotoxicity in response to HIV as neurons are unable to be infected [17,18,86]. Although the essential role of macrophages and microglia has been recognized for over 20 years, the mechanisms that underlie neurotoxicity are still poorly understood. In almost all studies neurotoxicity in response to HIV proteins and macrophage conditioned medium has been correlated with an abnormal rise in intracellular calcium. Most studies have shown that the N-methyl-D-aspartate (NMDA) glutamate receptor contributes to the calcium rise although voltage-gated calcium channels and IP3 receptors have also been implicated. Some studies have emphasized the delayed and sustained rise in neuronal calcium levels [87,288] showing that the long term elevation of calcium is specifically associated with and precedes the formation of dendritic beading and neuronal injury [75,92,289]. In previous work done from our lab conditioned medium from macrophages infected with feline immunodeficiency virus caused calcium dysregulation in cultured feline neurons and varicosity (beading) formation along the neuronal processes[90,288]. Though this was an infection model, other studies demonstrated that gp120 binding independent of infection was sufficient to trigger secretion of neurotoxins leading to calcium dysregulation and varicosities [86,87]. Similar

neurotoxicity was seen using cerebrospinal fluid from HIV infected patients verifying the in vivo relevance of this toxicity [290,291]. Consistent with previous data, human monocyte derived macrophages exposed to HIV_{ADA} also secreted unknown neurotoxins in culture that were damaging to neuronal cells as assessed by a delayed rise in calcium and the formation of varicosities. Since this is a well-established and reliable correlate of HIV-associated neuropathogenesis, the assessment of neurotoxin secretion was used as a disease relevant functional endpoint for the studies in this thesis. Surprisingly, studies in this thesis demonstrated that secretion of the neurotoxic factors from macrophages was dependent on CXCR4 activation and not CCR5. This was counter intuitive because the virion used in our studies preferentially targets the CCR5 HIV co-receptor and CCR5-preferring virus is most commonly seen in brain. This data suggested that HIV induced macrophage activation via the CXCR4 receptor is necessary for toxin production but the virus strain was not of primary importance.

Much attention on macrophage activation has been given to their ability to secrete both pro-inflammatory and anti-inflammatory proteins dependent on their external cues. To understand how HIV induced macrophage activation influenced secretion of pro-inflammatory and anti-inflammatory cytokines, we analyzed the conditioned medium from HIV stimulated macrophages and evaluated patterns for M1 or M2 activation. M1 markers assessed were TNF-α, IL-12, p70, IL-6, IL-23 IFN-γ, IL-1α,β and IP10. M2 markers were IL-4, IL-10, IL-13, TGF-β (1, 2, 3 & 5), and MCP-1. In our secretome analysis we did not see a clear M1 or M2 response to HIV. We also did not see HIV-induced changes in CD16 or CD163 cell surface expression, markers of M1 and M2 respectively. However, this classification system does not take into account the many key actions of macrophages. In

addition, in spite of the widespread use of markers of "inflammation," there is little evidence to show that they contribute directly to neuronal degeneration, and their release has only occasionally been directly linked to deleterious functional outcomes.

Changes in actin structures and calcium spiking in macrophages correlate with toxin production

When macrophages were challenged with HIV, changes in cell morphology were seen that indicated a shift toward the expression of podosomes. Previous studies showed that the migration of macrophages and infiltration into tissue in response to HIV-Nef required the formation of actin rich podosome foci. These Nef-induced podosomes were clustered in rosettes or along the outline of the cells known as belts or podonuts and the overall expression of podosome number and stability within the cell was dependent on HIV stimulation [244]. In our study, untreated cultured macrophages possessed a mix of cells with actin rich podosome structures, ruffled structures or neither of these actin specializations. During HIV_{ADA} exposure in culture, the number of podosome bearing cells and podosome clusters increased. This induction of podosomes was correlated to neurotoxin production while cultures with more ruffled bearing cells had significantly less neurotoxin production. Blocking CXCR4 activity during HIV stimulation increased the number of ruffled cells in culture and reduced the density of podosomes found in the cells. Ironically, the HIV co-receptor CCR5 had no effect on the development of these morphological specializations. These data indicated that neurotoxin and podosome formation during HIV exposure is dependent on CXCR4 activation and not CCR5.

Changes in actin structures have been linked to calcium influx in macrophages. In the RAW 264.7 mouse macrophage cell line, spontaneous polarization and migration were associated with influx of extracellular calcium at the leading edge of movement. The calcium increase was necessary for maintenance of F-actin and membrane ruffles via a PI3K-dependent pathway [208]. Other studies correlated calcium spiking in macrophages to ruffled structures and increased phagocytosis [205-207]. In our studies, hMDM with more ruffled structures had increased calcium spiking and more phagocytic activity than non-ruffled cells which was negatively correlated with neurotoxin production. The Collman group reported elevated intracellular calcium in macrophages after stimulation with HIV JFRL (R5 tropic) and IIIB (X4 tropic) virons [271] although the nature of the calcium response was not documented. Though we saw an acute increase in calcium influx, the overall calcium buildup was minimal and calcium spiking was suppressed with HIV exposure. This was consistent with a reduction in ruffled cells in culture. This data indicated that modification in specific actin rich structures can be useful in identifying toxic phenotypes in macrophages. As discussed below this dissertation has discovered that neurotrophin signaling also plays a prominent role in the differentiation of these structural phenotypes.

Macrophages express neurotrophin receptors

Though the idea of immune cell interactions with neurotrophins was postulated almost 20 years ago [116], not much work had been done to understand the interplay of these systems. The current data documenting expression of $p75^{NTR}$ and Trk receptors on immune cells is inconsistent, often only measures gene transcripts or identified in pathology tissue where it was hard to distinguish expression on various cell types. The data in this thesis

assessed the expression of neurotrophin receptors on isolated macrophages and monocytes and for the first time determined the level of expression and cellular localization. TrkA and the $p75^{NTR}$ were found in higher quantities than other neurotrophin receptors under normal conditions on both monocytes and monocyte derived macrophages. Low amounts of TrkB were seen but we found no evidence for TrkC receptors on macrophages. Reports for TrkB and TrkC expression on macrophages have been citied in lymphoid tissues and microglial cells [179,292,293]. It is possible that these receptors are expressed in higher quantities in various tissues and may be upregulated under pathologic conditions. In this study TrkB expression during HIV stimulation was not assessed nor was the presence of its natural ligand, BDNF. Although $p75^{NTR}$ and TrkA were more robustly expressed, it does not diminish the need for better understanding of BDNF/TrkB signaling in macrophages. Several reports indicate that BDNF may be neuroprotective in HIV models of neuropathogenesis, and its upregulation was associated with protection in response to memantine administration in a SIV model [98,154,155,294]. Though its neuroprotective effects focused on neuronal cells in these studies, TrkB's appearance in endosome like structures in our studies suggests a potential role for signaling in macrophages that needs to be understood. However, due to the abundance of the $p75^{NTR}$ and TrkA found on hMDMs, this thesis focused on the potential regulation of macrophage activation with their endogenous ligands proNGF and NGF respectively.

ProNGF vs. NGF

With recent interest in the imbalance between pro vs mature neurotrophins during neurodegenerative diseases, this study for the first time investigated the role of proNGF on

human macrophages and how it differs from mature NGF signaling. Unlike other neurotrophin receptors, the $p75^{NTR}$ lacks a catalytic domain and requires the assistance of other receptors to carry out signaling. The two common classes of receptor interactions with the $p75^{NTR}$ are the neurotrophin receptors (TrkA, B and C), the sorting protein sortilin, and the Nogo receptor. Heteromeric interactions of the $p75^{NTR}$ with Trk receptors increases the affinity of the mature neurotrophin/Trk interaction by about 100-fold and enhances pro-survival and growth signaling [295,296] while pro-neurotrophins bind with high affinity to the sortilin-$p75^{NTR}$ complex and initiate cell death signaling[134]. Results from this thesis showed for the first time that the $p75^{NTR}$ co-localized with both TrkA and sortilin in human monocyte derived macrophages providing an environment conducive to signaling with mature NGF and proNGF.

In addition to the expression of neurotrophin receptors, activated microglia/macrophages can release neurotrophins [297,298] providing the capacity for autocrine feedback although only a few studies have provided functional data evaluating NGF production by macrophages. NGF has been shown to increase cell viability in two microglia cell lines [299] and increased the expression of FC□ receptors and phagocytosis on murine macrophages [300,301]. Consistent with these reports, our studies showed that NGF stimulation of serum deprived hMDM increased cell viability in a dose dependent manner that was mediated by TrkA signaling. Under normal conditions, NGF increased ruffling of the membrane, phagocytic activity and calcium spiking in hMDMs. Similar to the Samah et.al. study, results from the protein arrays and flow studies in Chapter 1 showed NGF did not increase TNF secretion or the expression of M2 surface markers CD163 and CD206.

Conditioned medium from these cells showed low levels of neurotoxicity, indicating that macrophage activation by NGF does not produce a neurotoxic phenotype.

ProNGF stimulation, however, resulted in a vastly different phenotype than NGF. This comparison was important because of our demonstration of robust $p75^{NTR}$ expression and the absence of previous studies of proNGF signaling in macrophages/microglia. Responses to proNGF are of particular importance since increases in proNGF secretion from macrophages/microglia have been described during LPS treatment, brain injury and neurodegenerative diseases [302,303]. Minocycline, a broad anti-inflammatory modulator, inhibited proNGF production from microglia during spinal cord injury [304] while direct administration of proNGF into the hippocampus of mouse brain increased activation of microglia as measured by increased CD11b/BrdU co-stains and more ramified bushier morphologies of microglial-like cells in brain tissue [305]. Though proNGF has been associated with apoptosis through interactions with the $p75^{NTR}$ and sortilin, we did not see any cell death despite an increase in sortilin expression. In this context, it is important to note that sortilin also functions as a sorting protein and is found mostly within late endosomes derived from the trans-Golgi network. Sortilin expression has been seen on microglial mouse cell lines and is involved in phosphorylation of AKT, migration and increased appearance of actin flilopodia and increased MIP-2, MCP-1, IL-1β and TNF-α secretion during stimulation with sortilin's other ligand, neurotensin [211,212,306]. Consistent with this data, both NGF and proNGF stimulation increased phosphorylation of AKT in macrophages in our studies. Actin rich structures were remarkably changed with proNGF stimulation as shown by an increase in the number of cells expressing podosomes. These podosomes were often polarized to one quadrant of the cell which is consistent with

increased migration seen in these cells. ProNGF also suppressed calcium spiking in macrophages and neurotoxin production was increased compared to NGF stimulated cells. Taken together these data indicate that proNGF and mature NGF regulate macrophage activation differentially and raise the possibility that neurotrophin intervention may be able to control inflammation in response to HIV.

The molecular interactions that control neurotoxin production by macrophages are still poorly understood. The phenotype induced by proNGF stimulation of macrophages was similar to that seen in response to HIV exposure, resulting in podosome formation and polarization, decreased calcium spiking and increased neurotoxin production. Importantly, the induction of podosomes and to a lesser extent neurotoxin production by HIV was dependent on $p75^{NTR}$ signaling while TrkA signaling was involved in suppressing neurotoxicity. Also, HIV exposure increased the co-localization of both $p75^{NTR}$/TrkA and p75/sortilin, suggesting that the macrophages respond to the pathogen by increasing the availability of interactions between these receptors. The similarities seen between HIV and neurotrophin stimulation and the ability of HIV to foster neurotrophin signaling suggested that neurotrophin signaling may modulate HIV effects in macrophages. To determine the relationship between HIV and neurotrophin signaling we investigated the interactions of the neurotrophins, proNGF and NGF, with HIV induced macrophage activation.

Since proNGF has been strongly linked to apoptosis in various cells, we first explored the possibility that HIV and proNGF might synergize to induce the death of the MDM. This was not only important mechanistically but was an important control for investigation of the effects of proNGF. Although we saw no cell death of MDM in culture with proNGF or HIV stimulation alone, a very small increase in apoptotic nuclei was seen when the MDM were

co-stimulated with proNGF and HIV suggesting a small synergistic effect. However, although proNGF and HIV alone each had similar effects on the suppression of calcium spiking in macrophages, co-stimulation had no synergistic effect. This suggested that proNGF and HIV may be acting through common pathways to suppress calcium spiking. Similarly, one hour co-stimulation of proNGF and HIV had no synergistic effect on HIV induced podosome formation although an increase in the polarization of podosomes was seen. Consistent with this observation, co-stimulation with proNGF + HIV increased migration in these cells. Lastly, co-stimulation of proNGF and HIV exacerbated the HIV induced neurotoxicity caused by macrophages. This data indicated that while proNGF did not have a synergistic effect on calcium spiking it did cause a more damaging phenotype in macrophages during HIV exposure which was strongly associated with increases in the number of podosome bearing cells.

Macrophage phenotypes in response to co-stimulation with NGF and HIV contrasted sharply with HIV treated cells as well as HIV + proNGF. NGF was able to reverse the suppression of calcium spiking by HIV. This was consistent with NGFs ability to increase spiking in macrophages alone and indicated that the effect of NGF overrode the effects of HIV. However, as calcium spikes have been correlated with phagocytosis [205], it was surprising that we saw a reduction in phagocytic activity with co-stimulation of NGF and HIV. One possible reason for this is that phagocytosis is also correlated to membrane ruffling and we did not see an increase in membrane ruffling with NGF+HIV co-stimulation. NGF decreased podosome expression in response to HIV and increased the number of cells in culture that lacked both podosomes and ruffles which may represent a “transitional” phenotype. In concert with these observations, co-stimulation of hMDMs with NGF and

HIV partially suppressed HIV induced neurotoxicity indicating that NGF may have the potential to interfere with HIV signaling and suppress the development of the toxic macrophage phenotype.

Neurotophin regulation is mediated through interactions with CXCR4

The strong relationship between the effects of HIV and proNGF, the ability of NGF to reverse many of the changes induced by HIV and the co-localization of all receptors in similar domains all supported the possibility of interactions between these receptors. In an effort to understand how NGF may be interfering with HIV signaling we investigated potential direct interactions with the HIV co-receptor, CXCR4. We chose this co-receptor instead of CCR5 because our data showed that CXCR4 and not CCR5 were involved in neurotoxin production and the formation of podosomes. Also, conflicting literature has suggested that NGF sustains macrophage viability and HIV replication in macrophages [174] while others suggested that it inhibits HIV replication while enhancing CXCR4 but not CCR5 expression [171]. NGF has also been shown to increase chemotaxis mediated by it's the CXCR4 ligand SDF-1 in part due to an increase in receptor expression in both untreated and HIV infected hMDM [175,176]. However these studies did not explain how NGF may be mediating CXCR4 expression and activity. Moses Chao's group introduced the idea that Trk receptors can be transactivated by GPCRs [276-280], and transactivation of CXCR4 by the insulin-like growth factor-1 receptor has also been reported in human breast cancer cells [281]. Also, transcriptional regulation of CXCR4 has been cited by a number of signaling molecules including growth factors TGF-1β[307], basic fibroblast growth factor[308,309], vascular endothelial growth factor[309] and epidermal growth factor[286] while

inflammatory markers tumor necrosis factor-□[310] interferon-□[310] and IL-1□[310] has been shown to negatively regulate CXCR4 expression. These studies set precedence for possible regulation of CXCR4 by a variety of factors, but the nature of these interactions was not determined. In our studies we did not see an increase in total CXCR4 with HIV or neurotrophin signaling. This discrepancy with previous studies that have shown increased CXCR4 expression in the cell surface of hMDMs may be attributed to the whole cell versus cell membrane expression of the receptor. In our studies we saw that total CXCR4 is diffusely expressed in hMDMs but once activated, phosphorylated CXCR4 is sequestered in the outer extremities of the cell. Though we did not address membrane versus cytosolic CXCR4 our studies, it is possible that NGF stimulation increases CXCR4 receptor expression on the cellular membrane without altering total CXCR4 cellular expression. Many studies have shown that CXCR4 engagement with its natural ligand causes phosphorylation of the receptor [284]; however, it is unclear in the literature if phosphorylation of CXCR4 occurs after engagement with HIV and no study has looked at the ability of neurotrophins to stimulate phosphorylation of CXCR4. In our study we showed that HIV induced a small increase in CXCR4 phosphorylation. This small increase in phosphorylation by HIV suggested that phosphorylation of CXCR4 is not necessary for toxin production. In preliminary observations, the toxicity of conditioned medium correlated with reduced CXCR4 phosphorylation. Consistent with this, co-stimulation with NGF but not proNGF increased this phosphorylation in a TrkA dependent manner. These data suggest that neurotrophin signaling may be able to regulate CXCR4 activation states. The likelihood of direct regulation of CXCR4 by the neurotrophin receptors was demonstrated in Chapter 3 by co-immunoprecipitation of the receptors. Both TrkA and $p75^{NTR}$ were found in complexes

with CXCR4. Consistent with the inability of HIV and the neurotrophins to alter total CXCR4 expression, HIV had no effect on TrkA and $p75^{NTR}$ complexes with total CXCR4. However, as stated earlier, pCXCR4 expression was sequestered in the outer extremities of hMDM in similar domains as neurotrophin receptors. Phospho-CXCR4 often overlapped with both TrkA and $p75^{NTR}$. HIV reduced the regional overlap between pCXCR4 and the neurotrophin receptors. While co-stimulation had no effect on pCXCR4/TrkA overlap, proNGF decreased pCXCR4/ p75NTR foci in a synergistic manner with HIV. In contrast, NGF co-stimulation with HIV restored pCXCR4/p75 overlap to control levels and partially increased pCXCR4/TrkA overlap. These data suggested an important role in pCXCR4 and neurotrophin interactions during NGF stimulation. However, the partial increase in pCXCR4 and TrkA may explain why we only see partial suppression of neurotoxicity by NGF in our studies.

The G protein-coupled receptor kinase (GRK) family has been implicated in the phosphorylation of CXCR4. The exact family member mediating CXCR4 phosphorylation is unknown and currently there are no good specific inhibitors to identify the exact member. Nevertheless, GRK2 has been suggested since its overexpression increased SDF-1 mediated internalization of CXCR4, a step in CXCR4 activation immediately following phosphorylation [284]. To understand GRK2 involvement in our studies we showed that GRK2 formed complexes with CXCR4. HIV reduced the amount of GRK2/CXCR4 complexes whereas NGF co-stimulation increased GRK2 interaction with CXCR4. This supported the hypothesis that GRK2 may be the kinase responsible for phosphorylation of CXCR4 during NGF signaling. The ability of NGF to alter the HIV-associated phenotype may be through increased phosphorylation of CXCR4 whereas proNGF may be acting

through pathways common to HIV activation to create synergistic effects on macrophage neurotoxin phenotypes.

What is the neurotoxin?

The hunt for the neurotoxin has been ongoing since the early 90's when Dana Giulian and Lynn Pulliam showed that neuronal damage was caused by factors secreted from HIV infected macrophages [18,276]. Since then many studies have tried to identify the neurotoxin and factors such as quinolinic acid, arachidonic acid, nitric oxide, viral proteins and inflammatory cytokines have been suggested[85]. However, none of these factors have been shown to directly induce neuronal damage that recapitulates the effects of macrophage conditioned medium when assessed in vitro. In this study we provided the first comprehensive evaluation of cytokines and growth factors in the macrophage secretome as well as changes in response to HIV_{ADA}, proNGF and NGF. Overall, the pattern was similar to what we saw in our above experiments, with proNGF sharing many similarities with HIV and NGF stimulating a very different secretory profile that counteracted many of the effects of HIV. This was evident in that co-stimulation of macrophages with NGF in the presence of HIV normalized the expression of 58 out of 67 proteins (increased or decreased) secreted in response to HIV with a synergistic effect seen in only 2 proteins. In contrast, proNGF reversed changes in 24 out of 67 proteins but showed 11 synergistic effects with HIV. Thus, effects on protein secretion recapitulated the morphological and functional effects of proNGF and NGF alone and in combination with HIV.

To provide a better interpretation of macrophage functional phenotype, we used a bioinformatics tool (DAVID) to analyze the protein changes in the secretome during HIV exposure and other conditions. Six functional groups were identified: immune response, inflammatory response, defense response, chemotaxis, response to wounding and regulation of cell proliferation. Similar groupings were identified by DAVID when analyzing protein profiles from NGF or proNGF with or without HIV. These functional groups assigned by DAVID were consistent with the known functions of macrophages; however, they did not give the full picture of how vastly different the secretory profiles were in the various conditions. In a more thorough analysis of the secretome we grouped the significantly changed proteins into five families based on the DAVID analysis: growth factors, cytokines, chemokines, adhesion/MMP and other proteins. To improve the functional relevance of the protein array studies, the protein changes were correlated with the average level of calcium dysregulation seen under each stimulation condition. This helped to identify proteins whose changes could predict the neurotoxic outcome. Proteins that emerged as most relevant included proteins belonging to the angiogenesis family, the TGF beta family, select cytokines and the MMP family. These are discussed below.

Angiogenesis: Proteins regulating angiogenesis were most affected by proNGF, proNGF+HIV and HIV alone. We did not see changes in the angiogenesis family during NGF stimulation although studies have reported its positive role in angiogenesis [216]. The role of proNGF and HIV in angiogenesis is not well understood. $P75^{NTR}$ expression was upregulated in endothelial cells of type one diabetic mice and was involved in inhibiting neovascularization [253]. Although little is known about the significance of angiogenesis in

HIV infection, work on angiogenesis has been done in the context of Kaposi sarcoma, an angioproliferative mesenchymal cancer often seen in AIDS and other immune deficient patients. HIV Tat and nef have been shown to promote Kaposi sarcoma induced angiogenesis[250,251]. The anti-angiogenic factor thromposondin-1 inhibits HIV-Kaposi sarcoma induced angiogenic activity[252]. In our studies, thrombospondin and thrombospondin -1 were increased with HIV and angiostatin was reduced by HIV. ProNGF stimulation of macrophages increased the secretion of thrombospondin, thrombospondin-1, and angiostatin; however, during co-stimulation of proNGF and HIV, proNGF was able to restore the secretion of angiostatin. Co-stimulation with NGF and HIV reduced thrombospondin and thrombospondin-1, validating its role in angiogenesis. This suggested that HIV may have a potential role in providing factors to halt angiogenesis but combined therapies with neurotrophins during cancers such as Kaposi sarcoma might be fruitful. Differentiation of specific macrophage phenotypes have been implicated as a key event in the stimulation of angiogenesis and related tumor growth [311-313], and targeting macrophages for cancer therapy has been suggested [314,315]. By inducing suppressive or supportive macrophage phenotypes, the neurotrophin receptors may play an unrecognized role in the control of angiogenesis, introducing an important new area of investigation with high clinical significance for studies of tumorigenesis.

Other Growth factors: It is notable that growth factor secretion was changed more than any other family of proteins. This single observation greatly strengthens the growing recognition that pro-growth and pro-repair functions of macrophages are at least as important and perhaps more important than the destructive pro-inflammatory effects that have dominated the literature. In this category, the TGF and FGF families were most highly

represented. The profile of proteins altered by HIV and the neurotrophins introduced new proteins that were robustly expressed by macrophages such as the GDF subgroup of the TGF family. No studies exist on the role of macrophage-derived GDFs in HIV neuropathogenesis. In our study, secretion of many GDFs was changed with HIV or the neurotrophins. However, GDF3 was the only member of this family whose increase by HIV correlated strongly with toxicity. Only a few studies have examined these proteins in the context of other diseases. GDF15 is important for postnatal neuron survival in developing and lesioned neurons [316] and reduces macrophage accumulation at sites of inflammation [317], possibly via regulation of chemotaxis [318]. FGF-2 has been associated with inhibiting the angiotoxic effect of gp120 on endothelial cells lining the blood brain barrier [68] and was increased with co-stimulation of NGF+HIV in our study. The only FGF family members that correlated with neurotoxicity were FGF-9 which was increased with HIV and FGF-16, which was decreased. Not much work has been done on either FGF-9 or FGF-16 in macrophages or in the context of HIV. FGF-9 has been shown to be secreted from neurons and to support basal forebrain cholinergic neurons [254]. However more work needs to be done to understand the importance of these factors in the context of HIV neuropathogenesis.

Cytokines: Cytokines have been more extensively explored in HIV infection than any other protein group. MCP-1, MIP1α, MIP-1β, and MIP-2 upregulation has been reported in the CSF of patients with HIV infection or other neurodegenerative disorders [319,320] . Each of these proteins, excluding MIP-1α, was increased with HIV. In the protein array analysis MCP-1 was increased. MCP-1, MIP-1β, and MIP-2 secretory levels were restored to control levels or suppressed further with NGF co-stimulation. ProNGF stimulation in the presence or absence of HIV increased secretion of MIP-1b and MCP-1. Although proNGF stimulation

alone increased MIP-1β it did not have an additive effect on HIV-mediated release. It is also worth noting that although MIP1α was not increased by HIV stimulation, co-stimulation with NGF reduced its secretion to a level lower than controls. These data show that chemokine release is a prominent response to inflammatory challenges and also demonstrate a role for NGF in the suppression of chemokine secretion while proNGF exacerbates secretion in hMDM during HIV exposure. However, none of the above proteins correlated with calcium dysregulation. This is consistent with contradictions in the literature regarding their involvement in protection versus damage during disease. Thus, although these factors are increased during HIV exposure there is little evidence from studies in this thesis as well as other labs that they are directly involved in neurotoxicity.

MMPs: Few of the results from the protein arrays provided leads that might identify the protein(s) responsible for neurotoxicity. The strongest candidates from these studies were proteins within the MMP family. NGF increased MMP-7 and MMP-11 in the presence and absence of HIV but suppressed the release of MMP-9 by HIV. Support for the importance of the MMPs has come from studies showing their involvement in synaptic damage and remodeling.

MMP-7 was low in the medium of unstimulated cells and largely unaffected by HIV. However, it was increased by NGF. MMP-7 has been shown to be one of the enzymes that cleaves proNGF to NGF [255] and has also been implicated in positive synaptic remodeling by altering presynaptic terminals without affecting neuronal survival [224]. NGF's ability to increase MMP-7 secretion may be a feedback mechanism to support NGF signaling while also aiding in reconstruction of any damage caused to neurons during HIV exposure. A

potential positive influence of MMP-7 was supported by the negative correlation with toxicity.

MMP-11 secretion was also increased by NGF stimulation. Most of the work looking at MMP-11 in the central nervous system involves gliomas [256,257]. Its involvement in HIV neuropathogenesis is unclear, but the anti-angiogenic phenotypic switch with proNGF stimulation in macrophages found in this thesis suggests a potential relationship with MMP-11 that should be explored further.

One of the most striking observations from the protein array analysis was the increase in MMP-9, and tissue inhibitor of metalloprotease-1 (TIMP-1) in response to HIV. Both proteins were robustly expressed and had positive correlations to calcium dysregulation. The increase in both MMP-9 and its endogenous inhibitor TIMP-1 suggested that the hMDM constantly provide potentially deleterious and protective proteins at the same time in an effort to maintain tissue homeostasis. The balance between MMP-9 and TIMP-1 may be a crucial factor in the regulation of tissue damage and has been seen in other brain injury models including ALS [258-261]. Evidence for deleterious effects when the balance is changed was seen in the effects of proNGF which primarily decreased the secretion of TIMP-1, a result consistent with the increase in neurotoxicity of MCM after stimulation with both HIV and proNGF. MMP-9 is involved in activity dependent reorganization of dendritic spine and long term potentiation and has been implicated in abnormalities in spine morphology in a fragile X syndrome mouse model [223,262,263]. MMP-9 has also been associated with neuronal damage, including synapse degeneration and axonal demyelination in HIV infection and other neurodegenerative diseases [264-267]. In addition to the role of MMP-9 in the remodeling of the synapses during disease, part of MMP-9's damaging effects may be related

to its ability to metabolize NGF [268], creating a favorable environment for a high ratio of proNGF to mature NGF. The partially protective role of NGF could also be explained by its effects on MMP-9. NGF decreased MMP-9 and TIMP-1 in the presence of HIV suggesting its role in suppressing MMP-9 toxicity. MMP-9 was also increased with proNGF stimulation alone but had no effect on TIMP-1 secretion from macrophages. This increase in MMP-9 secretion may explain why conditioned medium from proNGF stimulated macrophages in the absence of HIV is neurotoxic. Finally, studies of macrophage invasion have demonstrated a strong relationship between podosomes and MMP-9 secretions, providing a link between the MDM phenotype and toxic activity [269,270]. The consistency of these observations makes MMP-9 a putative candidate for the neurotoxin. Indeed, preliminary results indicate that MMP-9 induces a gradual accumulation of calcium in neurons and MMP inhibitors reduce toxicity. Much work is needed to understand how MMP-9 may be involved in neuropathogenesis but it may serve as a potential biomarker or early indicator for disease prognosis. Consistent with this notion in Alzheimer and traumatic brain injury [267,321], neurotrophin intervention may be a way to control this aspect of macrophage activation.

Conclusion

This Dissertation has identified a neurotoxic phenotype in macrophages that include changes in actin structures, calcium activation and secretion of proteins that correlate with calcium dysregulation in neurons. Neurotrophin signaling strongly influence the phenotype in macrophages under normal conditions and in the presence of HIV. NGF drives a non-neurotoxic phenotype that increases calcium spiking and induces ruffles while reducing neurotoxin production in a TrkA manner. Its signaling through TrkA also increases phosphorylation of CXCR4 and fosters interactions between $p75^{NTR}$, TrkA and

phosphorylated CXCR4. These interactions lead to increased phosphorylation of CXCR4 which may be mediated through interactions with the G-protein kinase receptor GRK2. HIV and proNGF both create a neurotoxic phenotype in macrophages consisting of suppression in calcium spiking and the formation of polarized podosomes. The increased secretion of neurotoxins was $p75^{NTR}$ and CXCR4 dependent. Unlike NGF, proNGF and HIV had little effects on phosphorylation of CXCR4 and suppressed TrkA and $p75^{NTR}$ interactions with phosphorylated CXCR4, indicating that phosphorylation of the CXCR4 receptor is not necessary for neurotoxin production but instead suppresses HIV induced neurotoxicity. Of the proteins secreted from macrophages stimulated with HIV and/or proNGF, anti-angiogenic factors and MMPs were prominent. These profiles identify unique responses as well as potential sources of the neurotoxic activity. Novel interactions between the neurotrophin receptors and the HIV co-receptor CXCR4 were identified and open new therapeutic avenues for the regulation of macrophage function during HIV neuropathogenesis and other neurodegenerative diseases.

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