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Investigating Gene Expression Effects of Neuregulin-1 (NRG-1) and its Influence
on Response to Inflammatory Stimulation with LPS in Microglial Cells *In Vitro*

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Bioengineering

by

Catherine Jean Augello

March 2022

Dissertation Committee:
      Dr. Byron Ford, Chairperson
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                                    Committee Chairperson

University of California, Riverside

ABSTRACT OF THE DISSERTATION

Investigating Gene Expression Effects of Neuregulin-1 (NRG-1) and its Influence
on Response to Inflammatory Stimulation with LPS in Microglial Cells *In Vitro*

by

Catherine Jean Augello

Doctor of Philosophy, Graduate Program in Bioengineering
University of California, Riverside, March 2022
Dr. Byron Ford, Chairperson

Neuregulin-1 (NRG-1) is a potent, endogenous growth factor that has

shown promise in treating pathologies with a neuroinflammatory component,

but the mechanism through which NRG-1 treatment alters inflammation

remains an area of investigation. Studies using NRG-1 as a therapeutic

agent in stroke models have indicated that it has neuro-protective and anti-

inflammatory properties. In this work we evaluate the role NRG-1 plays in

altering inflammatory activation, especially in the context of neuropathology.

We examine the effects of NRG-1 on inflammation through *in vitro* application

of NRG-1 to microglia, the main immune cell of the central nervous system

(CNS).

To execute this work, we utilized the high through-put NanoString technology

to track gene expression changes and to identify the most prominent features

of NRG-1-induced alterations in the inflammatory state of activated SIM-A9

microglia *in vitro*. Stimulation with pro-inflammatory LPS and anti-
inflammatory IL-4 were used as benchmarks of canonical microglial
activation states to which NRG-1-induced changes were compared.
The most compelling results from this work are that NRG-1 enhances NF-kB
and apoptosis pathways that are triggered by pro-inflammatory stimulus and
are critical to cell survival. NF-kB influences microglial inflammatory states by
tweaking cell sensitivity to apoptotic signaling and increasing gene
expression within survival pathways. Specifically, NRG-1 pushes NF-kB
signaling towards the non-canonical arm which increases expression of
genes that encourage immune cell survival. This finding indicates a
potentially novel mechanism through which NRG-1 mediates anti-
inflammatory outcomes.

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OUTLINE

The opening chapter of this thesis provides background and a foundation for the development of this project. Chapter one traces the development of this project by introducing NRG-1 and the body of evidence that suggests NRG-1 improves tissue recovery at least partially through influence over inflammation in the central nervous system (CNS). The opening chapter also provides an overview of the neuroinflammatory setting and establishes the framework used within this work to study the influence of NRG-1 over neuroinflammation with respect to classical pro-and anti-inflammatory states within an *in vitro* model of microglial inflammation. Chapter 2 establishes the SIM-A9 microglial model of inflammation that is used in the remainder of this work. The chapter outlines the rationale behind choosing the SIM-A9 microglial cell line and confirms SIM-A9 cells as a competent model system to investigate NRG-1's influence on inflammation by showing ErbB expression and ligand-induced changes. The effect of NRG-1 on microglial inflammation is characterized in comparison to both classical, pro-inflammatory LPS and anti-inflammatory IL-4 stimulation through changes in the expression of established inflammatory biomarkers. Specifically, inflammatory state is identified though expression of well-understood biomarkers of inflammatory activation and pro- or anti-inflammatory state including nitric oxide (NO), interleukin-1 (IL-1), interleukin-10 (IL-10), interleukin-6 (IL-6), and Arginase 1 (Arg1).

Chapters 3 and 4 extend inflammatory state characterization from a few

markers to multidimensional gene expression data using high-throughput

NanoString® (NanoString Technologies Inc, Seattle WA) technology.

Chapter 3 utilizes the inflammation gene panel (Mouse Inflammation Panel,

285 genes) to examine how NRG-1 alters gene expression within LPS-

induced inflammatory cytokine signaling cascades and how NRG-1 changes

compare to those induced by anti-inflammatory stimulus IL-4 acting on LPS-

induced gene expression. Studies in Chapter 4 extend this work beyond

inflammatory genes into neuropathology (Neuropathology Panel,757 genes)

widening the scope of gene expression data to additional pathways

associated with neuroinflammation and neuropathology.

CHAPTER 1 INTRODUCTION AND SCOPE OF WORK

OVERVIEW AND OBJECTIVES

Previous *in vitro* and *in vivo* studies from our lab and others have established that NRG-1 can reduce levels of pro-inflammatory cytokines (Xu, Jiang et al. 2004, Simmons, Surles-Zeigler et al. 2016). When used therapeutically in models of neuroinflammatory conditions ranging from stroke (Xu, Jiang et al. 2004) to nerve agent (Li, Lein et al. 2015) exposure to malaria (Solomon, Wilson et al. 2014, Liu, Solomon et al. 2018), NRG-1 treatment has been reported to minimize neuronal loss and mitigate inflammatory responses. In addition, previous work in our lab has shown that NRG-1 stimulation can reduce pro-inflammatory cytokine production by microglia/macrophages using the N9 microglial cell line (Simmons, Surles-Zeigler et al. 2016). While NRG-1 has been characterized as an anti-inflammatory agent, the mechanisms and exact scope of these effects are not well-defined. In this work, we will induce pro-inflammatory activation in SIM-A9 microglia through LPS stimulation and evaluate how NRG-1 exposure alters gene expression, especially in comparison to the gene expression changes resulting from exposure to the known anti-inflammatory factor IL-4.

Our objective is to use gene expression changes to identify the most prominent features of NRG-1-induced alterations in the inflammatory state of activated SIM-A9 microglia. This may provide additional context to suggest pathways and mechanisms through which NRG-1 mediates the anti-inflammatory outcomes reported in previous publications. Overall, we expect

this work will provide greater insight into the influence of NRG-1 on neuroinflammation.

As will be shown later, evidence points towards NRG-1 treatment having inflammation modulating effects. This is based on significant but limited number of inflammatory markers such as NO and cytokines IL-6 and IL-1β that were used to identify inflammatory activation within our experiments. There are numerous reports within literature that suggest NRG-1 may reduce pro-inflammatory activation in microglia. Previous work published by our lab (Simmons, Surles-Zeigler et al. 2016) established that pre-treatment with NRG-1 significantly reduced LPS-induced production of pro-inflammatory cytokine TNF-α and inflammatory activation marker IL-6 in N9 microglial cells. Similar observations were reported in experiments where pro-inflammatory stimulus was delivered concurrently with NRG-1, with Mencel et al(Mencel, Nash et al. 2013) reported that concurrent exposure to LPS and NRG-1 significantly reduced IL-6 production in the BV-2 cell line and Shahriary et al (Shahriary, Kataria et al. 2019) demonstrating higher doses of NRG-1 (above 50ng/mL) reduce proinflammatory cytokines, as well as pro-inflammatory surface marker CD86 and NO production in a primary isolated microglia *in vitro* . Further evidence that is often interpreted as indicating that NRG-1 exposure dampens pro-inflammatory activation is that NRG-1 reduced NO and superoxide production (Dimayuga, Ding et al. 2003). Kronenburg failed to see any indication of anti-inflammatory gene expression

increases within NRG-1 treated primary microglia in vitro (Kronenberg, Merkel et al. 2019).

I hypothesize that NRG-1 treatment will alter microglial inflammatory activation by dampening pro-inflammatory gene expression without triggering gene expression changes induced through IL-4-stimulation, which push microglia towards an anti-inflammatory state. Additionally, I plan to investigate how NRG-1 alters gene sets outside of inflammatory signaling that play a role in neuroinflammation and pathology.

THE ROLE OF MICROGLIA IN THE CNS

The microglial cell is the resident immune cell of the central nervous system (Ransohoff and Cardona 2010). These cells originate from hematopoietic progenitor cells in the yolk sac and migrate into the neural tube during embryogenesis (Ginhoux, Greter et al. 2010, Ransohoff and Cardona 2010, Lampron, Elali et al. 2013). Once in the brain, microglia establish a self-renewing population that does not require replenishment from circulating monocytes, distinguishing them from other tissue-resident macrophages which recruit bone-marrow derived cells (BMDCs) to maintain their numbers. While microglia are often understood as the resident macrophage of the CNS, these cells are unique in that they serve many functions in addition to their ability to phagocytose (Colton and Wilcock 2010). Under physiological conditions, microglia are often referred to as quiescent, naïve or "non-activated", but even in this state microglia are quite active. Microglia perform

many important homoeostatic functions, including providing trophic support for neurons, aiding in synaptic pruning, phagocytosing dead cells, engaging in self-renewal of their population, and surveilling other cells and the general environment (Hanisch and Kettenmann 2007, Gomez-Nicola and Perry 2015). Non-activated microglia have a distinctive, highly ramified morphology *in vivo* characterized by radially extending long, thin processes (Boche, Perry et al. 2013, Walker, Beynon et al. 2014). While not migrating long distances, *in vivo* imaging of intact mouse brains has shown that microglia are constantly moving, extending and retracting their processes every few minutes to seconds in order to probe or surveil the surrounding environment (Davalos, Grutzendler et al. 2005, Nimmerjahn, Kirchhoff et al. 2005). This state could be called the "surveillance" state as the microglia are acting as sentinels within the brain, on alert for anything out of the ordinary.

MICROGLIAL ACTIVATION

In response to disease or injury, microglia undergo a complex set of changes, referred to as activation. Activation can be understood as the transition from the homeostatic state to an effector state. Microglia express a wide array of pattern recognition receptors on their surface which are sensitive to unusual factors in the environment. The excitation of these surface receptors by pathogen or damage-associated molecular patterns (PAMPs and DAMPs, respectively) triggers activation (ElAli and Rivest 2016). Microglial activation *in vivo* has traditionally been identified by a

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distinct change in morphology which occurs within minutes of application of activating stimulus (Davalos, Grutzendler et al. 2005). The cellular processes retract, becoming shorter and thicker. Eventually the processes all but disappear and microglia take on an "ameboid" morphology (Boche, Perry et al. 2013). Morphological changes are accompanied by functional and behavioral changes. For instance, amoeboid microglia are able to easily migrate to areas of injury and phagocytose invaders or damaged cells (Walker, Beynon et al. 2014). In addition, surface receptors are upregulated, and cytokines are released.

Biomarkers of Microglial Phenotypes

Activated microglia are extremely heterogeneous, performing a multitude of functions during various stages of the inflammatory response. Most often microglia are characterized as "pro-inflammatory" or "anti-inflammatory", depending upon the nature of the cytokines the cell is excreting and the expression of specific cell surface markers (Ransohoff and Perry 2009, Martinez and Gordon 2014). Pro- or anti-inflammatory behaviors are complex and multifaceted concepts, characterized by the diverse functions microglia perform as they adopt various phenotypes in response to their environment. Activated microglia increase excretion of effector molecules. Pro-inflammatory microglia release cytotoxic factors to encourage cell damage while anti-inflammatory microglia release growth factors and extracellular matrix to aid in regeneration and repair. The factors that microglia release

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contribute to the extracellular environment and can also act as autocrine and paracrine signals to other microglia. Microglia detect their surrounding microenvironment through the receptors expressed on their surface (Kettenmann, Hanisch et al. 2011, Hickman, Kingery et al. 2013). These receptors change in response to different signals, which in turn determines the types of signals to which the microglia will be sensitive (Hickman, Kingery et al. 2013). The nomenclature and classification schema applied to microglia originated in the study of macrophages which was adopted from T cell research (Martinez and Gordon 2014, Ransohoff 2016). Starting in the 1980s, researchers found that stimulation with factors from T_H1 vs T_H2 T cells elicited opposing responses (Murray, Allen et al. 2014, Ransohoff 2016, Devanney, Stewart et al. 2020). In analogy to T cell nomenclature, these polar responses in macrophages were named M1 and M2, with M1 being induced by pro-inflammatory T_H1 cytokines and M2 induced through excitation with anti-inflammatory T_H2 cytokines (Murray, Allen et al. 2014, Ransohoff 2016). The concept of polarization was extended to microglia, as the resident phagocyte of this CNS. Classical (M1) activation is the induction of the pro-inflammatory phase of the immune response. This state is marked by the production and excretion of pro-inflammatory and toxic factors. M1 activation is induced by pro-inflammatory factors and characterized by high release of pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β and proteases (e.g. matrix metalloproteinase 9 (MMP9) and toxic intermediates

including NO, reactive oxygen intermediates (ROI), superoxide anion (O_2^-), and reactive nitrogen/oxygen species (N_2O_3, NO_2) (Colton 2009, Colton and Wilcock 2010, Orihuela, McPherson et al. 2016). The inflammatory molecules serve as messengers, calling additional immune cells to the area of injury while the cytotoxic agents attack pathogens. Unfortunately, cytotoxic agents can also cause undesirable and counter-productive inflammotoxicity in surrounding tissues. Alternative (M2) activation resolves the pro-inflammatory environment and leads to tissue repair, wound healing, and a return to homeostasis (Gordon 2003, Lynch 2009). Traditionally, alternative activation was conceived as a delayed part of the stereotypical immune response to the pathogen or injury (Martinez and Gordon 2014). This theory seemed to be bolstered by findings that some anti-inflammatory cytokines are released by classically activated microglia and act in an autocrine manner. Initiators of alternative activation are also released from other immune cells such as T_H2 regulatory T cells as well as immune-modulatory cells in the brain such astrocytes (Martinez and Gordon 2014) (Murray, Allen et al. 2014).

All M2 cell types outlined in the expanded paradigm are considered anti-inflammatory and repair oriented. However, it is argued that these various states play different roles in the immune response, are identified by different expression profiles, and induced through different conditions.

M2a or "alternative activation" is induced by IL-4 and IL-13 and involved in T_H2 response to allergies or killing and encapsulation of parasites. This type

of activation is associated with increased levels of phagocytosis, the production of growth factors such as insulin-like growth factor 1 (IGF-1) and anti-inflammatory cytokines such as IL-10 (Martinez and Gordon 2014, Walker and Lue 2015). M2b, also referred to as "type II alternate activation" or "mixed activation" microglia are characterized by the expression of M1 markers combined with production of M2 type cytokines. This state is induced by the ligation of immunoglobulin Fc gamma receptors (FcγRs) (including CD16, CD32 or CD64) by immune complexes on microglia stimulated (primed) by agonists of IL-1R and toll-like receptors (TLRs) (for example, IL-1β and LPS) (Martinez and Gordon 2014, Franco and Fernández-Suárez 2015). M2b type cells promote Th2 differentiation and humoral anti-body production (Franco and Fernández-Suárez 2015). More specifically, expression of IL-12 is downregulated while IL-10 production and MHCII expression increase. Other hallmarks of this activation state are increased expression of CD32 and CD64 (Walker and Lue 2015) and increased phagocytic ability. The M2c or "Acquired Deactivation" state is induced by uptake of apoptotic cells (Colton 2009), oxidized lipids and exposure to anti-inflammatory cytokines IL-10 and/or TGFβ and glucocorticoid (Colton and Wilcock 2010, Franco and Fernández-Suárez 2015) hormones. Generally alternate activation is identified by low levels of pro-inflammatory cytokines (IL-1, TNFα, IFNγ). However, the M2b phenotype

is unique in that it is characterized by high levels of inflammatory cytokine production as well as high IL-10 and low IL-12 expression (Gordon 2003).

Microglial Activation Depends Upon Stimulation

In the previous section, we outlined several activation states which can be identified through expressed biomarkers. However, distinct activation states are induced by different stimulations. In other words, stimulation with various immune reactive stimuli or combinations of stimuli will result in a different inflammatory activation state in microglia (Xue, Schmidt et al. 2014). The traditional polarization model of inflammatory activation has already been enlarged to encompass the variety of M2 anti-inflammatory states M2a-M2c(Martinez and Gordon 2014), or even M2d according to some proposed schema. Further *in vivo* and *in vitro* studies have found intermediaries or inconsistencies within inflammatory macrophage and microglial populations (Fenn, Hall et al. 2014, Ransohoff 2016, Zhang, Bailey et al. 2016) leading many researchers to propose a spectrum model of activation as opposed to the older polarization model. In 2013 macrophage researchers at the International Congress of Immunology recommended nomenclature defined by stimulation, for example M(LPS) or M(IL-4)(Murray, Allen et al. 2014) (Orihuela, McPherson et al. 2016) to replace the M1/M2a-d system entirely. This both reflects the importance of stimulation in directing inflammatory state and acknowledges the wide range of unique and specific activations acquired subsequent to exposure to inflammomodulatory stimuli.

LPS as a Pro-Inflammatory Stimulus

Lipopolysaccharide (LPS) is a component of the cell wall of gram-negative bacteria and one of the most ubiquitous and well-studied pro-inflammatory molecules, often used in models of bacterial (or non-sterile) infection. LPS is part of the class of molecules called danger-associated molecular pattern (DAMPs). It signals through the toll-like receptor 4 (TLR4).

LPS is considered pro-inflammatory because it triggers gene expression changes in cells which lead to increased release of cytokines and chemokines to attract additional immune cells to the area and the production of cytotoxic factors (Chhor, Le Charpentier et al. 2013, Murray, Allen et al. 2014, Lively and Schlichter 2018). Some products of LPS-induced gene expression trigger the removal of TLR4 from the membrane or reduce gene expression of their own catalyzing enzymes, resulting in desensitization of microglia to additional pro-inflammatory signaling over time. Desensitization represents one avenue for resolution of inflammation, but often is not enough on its own. Instead, the influence of anti-inflammatory molecules is often crucial for resolving inflammation.

ANTI-INFLAMMATORY STIMULI AND RESPONSES
Anti-inflammatory is the opposite of pro-inflammatory. Anti-inflammatory stimuli often work by directly reversing gene expression induced by pro-inflammatory agents. However, anti-inflammatory effects are often defined as a reduction in pro-inflammatory response (Liu, Liu et al. 2016).

IL-4 as an Anti-Inflammatory Agent

One such anti-inflammatory agent is the cytokine interleukin-4 (IL-4). IL-4 is a canonically anti-inflammatory stimulus used to induce alternative activation in macrophages, and by extension microglia (Butovsky, Ziv et al. 2006, Colton and Wilcock 2010, Murray, Allen et al. 2014). IL-4 is widely regarded as a strong anti-inflammatory treatment based upon a myriad of studies in macrophages in which IL-4 administration concurrently with or slightly following pro-inflammatory stimulus results in reduced production of pro-inflammatory cytokines. More specifically, IL-4 is associated with the M2a pattern of activation, defined by release of anti-inflammatory molecules and contributing to tissue repair (Chhor, Le Charpentier et al. 2013, Latta, Sudduth et al. 2015). IL-4 and the closely related IL-13 act as ligands binding to their own receptor, transducing signaling through Jak1 and Jak3, which in turn triggers nuclear translocation of STAT6 and induction of increased levels of arginase 1 (Arg1) and SOCS1 as well as the membrane-bound receptors CD206 (mannose receptor, Mrc1) and CD163 (scavenger receptors) (Nelms, Keegan et al. 1999, Gadani, Cronk et al. 2012, Quarta, Berneman et al. 2020). It is also reported that M2a macrophages release the cytokine IL-10 (Murray, Allen et al. 2014, Xu, Wang et al. 2017), and IL-4 induced increase in IL-10 expression has been reported in some microglial cell lines (Pei, Wang et al. 2017, Zhang, Li et al. 2017).

Because IL-4 is recognized for its anti-inflammatory activity, groups have attempted to use it as a treatment against inflammation (Latta, Sudduth et al. 2015, Zhao, Wang et al. 2015, Rossi, Cusimano et al. 2018). Outcomes of experimental application of IL-4 have had mixed success. In some cases IL-4 application resulted in reduced total inflammation but negative functional outcomes.

Neuregulin-1 as a Treatment for Inflammation in Microglia

Another endogenous molecule used to treat inflammation is neuregulin (NRG-1). Our lab has published findings that NRG-1 has anti-inflammatory properties when delivered as a treatment for ischemic stroke in pre-clinical rodent models (Xu, Jiang et al. 2004, Xu, Ford et al. 2005). However, the unique and specific features of NRG-1 stimulation and subsequent induced inflammatory state in microglial cells have yet to be explored. A future challenge will be precisely and accurately defining NRG-1 induced biomarkers indicative of changes NRG-1 influences within pro-inflammatory response. A starting point to define NRG-1 activation state in relation to known gene expression changes acquired following stimulation with pro-inflammatory stimulus LPS and in comparison to how anti-inflammatory stimulus IL-4 alters LPS-induced gene expression. The following section provides a brief overview of NRG-1. NRG-1's anti-inflammatory properties are not limited to microglia.

NEUREGULIN-1 BACKGROUND

The neuregulins (NRGs) are a family of EGF-like signaling molecules that serve as ligands to ErbB transmembrane tyrosine kinase receptors, mediating cell-cell cross-talk that is critical for the development, maintenance and repair of many systems in the body, including the nervous system, cardiac and mammary tissues and other organ systems (Buonanno and Fischbach 2001). There are four NRG-1 genes, with NRG-1 being the most widely studied. Structurally, NRGs consist of extracellular immunoglobulin G or cystine-rich and EGF domains, as well as a transmembrane domain and c-terminal cytoplasmic tail region. NRG-1 can be further categorized into six types (I-VI), identified by a unique domain at the N-terminal side of the protein. Types I and II are cleaved from the membrane to act paracrine signals while type III is also cleaved from the pro- form but remain membrane-bound to serve as juxtracrine signals.
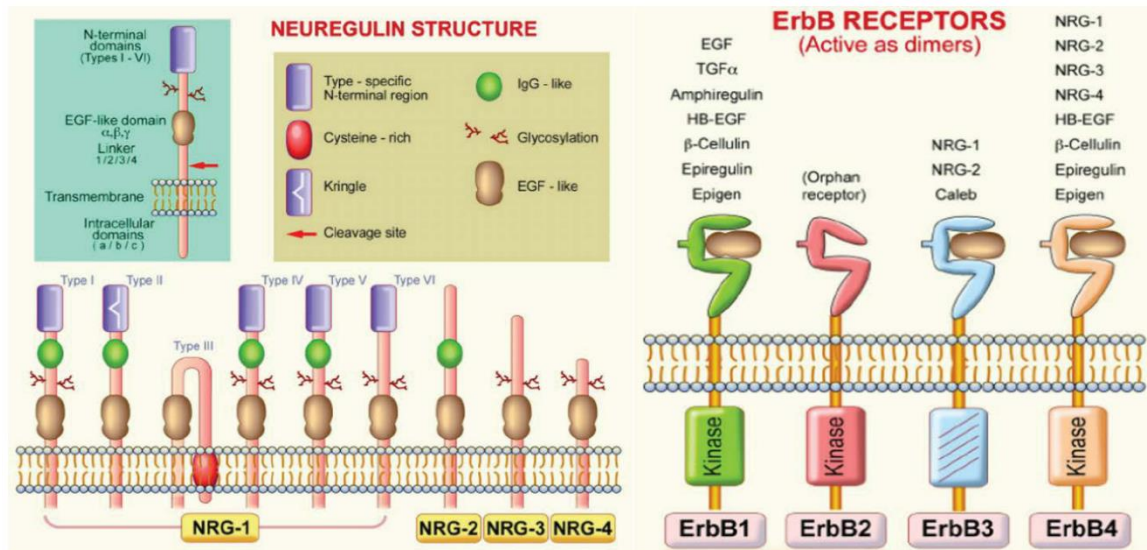
Figure 1-1: Forms of NRG-1 and their receptor, the ErbB family
Schematic of NRG structure and receptor compatibility. Reproduced from Cespedes et al. Int J Brain Disord Treat 2018, 4:024. (Cespedes, Liu et al. 2018)

NRG-1 binds receptors of the ErbB family through direct contact between the receptor and its EGF-domain. NRG-1 binding induces homo and heterodimerization of receptor monomers followed by phosphorylation which activates intracellular signaling cascades, ultimately inducing some specific cellular response. ErbB3 and ErbB4 are the dominant ErbB receptors expressed in the CNS, and *in vitro* experiments show NRG-1 preferentially binds ErbB3 and ErbB4 over EGFR or ErbB2 (Carraway, Weber et al. 1997). However, ErbB3 lacks an active intra-cellular domain, therefore NRG-1 signaling via ErbB3 must occur though heterodimers with other ErbBs. *In vivo* studies have shown that heterodimers of ErbB2 paired with ErbB3 or ErbB4 act as functional NRG-1 receptors (Cespedes, Liu et al. 2018). In addition, *in vitro* methods have identified ErbB4 homodimers as competent

NRG-1 receptors (Carpenter 2003) and ErbB4 homodimers are able to trigger intracellular signaling (Yarden and Sliwkowski 2001, Murphy, Krainock et al. 2002). Taken together, this indicates that the potential for NRG-1 signaling in the brain will be highly dependent to the levels of ErbB4 expression, as the dominant ErbB receptor present in neural tissues. These ErbB dimers signal through the mitogen-activated protein (MAP) kinase, phosphatidyl-inositol-3-kinase (PI3-K/Akt) (Liu, Solomon et al. 2018) and canonical and alternative NF-κB pathways (Simmons, Surles-Zeigler et al. 2016).

Evidence that NRG-1 Alters Inflammation

Neuregulin signaling plays well documented roles in many systems, notably during development but remains an important player throughout adulthood. In mature organisms, both *in vitro* and *in vivo* evidence support a connection between NRG-1 and inflammation. Strong evidence from myriad *in vivo* models indicate that NRG-1 treatment has an anti-inflammatory effect, both systemically and within the central nervous system (Xu, Jiang et al. 2004, Xu, Ford et al. 2005, Li, Lein et al. 2015, Simmons, Surles-Zeigler et al. 2016). NRG-1 is expressed endogenously on several cell types including neurons and microglial cells. Notably, this endogenous expression of NRG-1 (Parker, Chen et al. 2002) and its receptor ErbB4 are upregulated in response to CNS injury such as ischemic stroke (Xu and Ford 2005), traumatic brain injury (TBI) (Erlich, Shohami et al. 2000) specifically within the area of tissue

damage, suggesting that NRG-1 signaling is induced by and interacts with immune cells. When given in rodent middle cerebral artery occlusion (MCAO) models of ischemic stroke, NRG-1 treatment reduces a suite of ischemia-induced pro-inflammatory gene expression (Xu, Ford et al. 2005), including the decrease of clinical stroke biomarkers such as the pro-inflammatory cytokine IL-1β (Xu, Jiang et al. 2004). NRG-1 also prevents microglial activation following MCAO as measured by ED-1 staining (Xu, Jiang et al. 2004). NRG's ability to modulate and mitigate inflammation has been reported in a model of organophosphate (nerve agent) exposure, in which treatment with NRG-1 reduced neuro-inflammatory injury (Li, Lein et al. 2011).

NRG-1 is Neuroprotective and Anti-Inflammatory in Spinal Cord Injury

NRG-1 is acutely and chronically downregulated following spinal cord injury (SCI). In a rat model of compressive SCI, immediate addition of exogenous NRG-1 enhances a neuroprotective immune phenotype characterized by increased IL-10 and Arg1 expression (Alizadeh, Dyck et al. 2017). NRG-1 treatment also resulted in complementary reduction in proinflammatory factors such as IL-1β, TNF-α, MMP-9, MMP-2, and NO production globally at the site of injury. NRG-1 also reduced astrogliosis and scar formation by reducing injury-induced chondroitin sulfate proteoglycans, which make up the glial scar and inhibit regeneration. NRG-1 reduced expression of the TLR accessory protein MyD88, which may indicate reduced TLR mediated effects.

In addition, NRG-1 and phosphorylated Erk1/2 and STAT3. Erk1/2 activation can induce IL-10 release from microglia/macrophages, and IL-10 further mediates reduced proinflammatory cytokine production via STAT3 activation.

NRG-1 Effects in Models of Multiple Sclerosis and ALS

In demyelinating lesions, NRG1 promoted pro-regenerative inflammatory activation as characterized by IL-10 production (Kataria, Alizadeh et al. 2018). NRG-1 contributed to differentiation of neural progenitor cells into remyelinating oligodendrocytes as well as remyelinating Schwann cells. Anti-inflammatory activated microglia/macrophages have improved myelination. NRG1 delivery to the neuromuscular junction however, improved outcomes, illustrating that in ALS, NRG1 has very different outcomes dependent upon which cell types are mediating the NRG-1 signal.

Schizophrenia is a developmental, inflammation associated disorder that has been linked to specific genetic mutations in the NRG-1 gene (Marballi, Quinones et al. 2010, Zhang, Cui et al. 2019). In studies tracking human genetic variation within the NRG-1 protein, mutations in NRG-1 transmembrane domain result in altered cytokine levels (IL-6, IL-1β, TNF-α, IL-12p70, IL-10) in plasma of individuals of families carrying the NRG-1 mutation that is linked to schizophrenia and an increase in proinflammatory cytokine IL-6 and decrease in anti-inflammatory IL-4 mRNA levels in cultured cells (Marballi, Quinones et al. 2010). In addition, mice with a NRG-1 knockout develop schizophrenia-like conditions which is associated with

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immune system dysregulation and overexpression of proinflammatory

cytokines (Marballi, Quinones et al. 2010).

Anti-inflammatory Influence of NRG-1 on Microglia in vitro

The ability of NRG-1 to directly regulate and alter microglia has been

examined and more specifically studied *in vitro* (Dimayuga, Ding et al. 2003,

Calvo, Zhu et al. 2010, Mencel, Nash et al. 2013, Simmons, Surles-Zeigler et

al. 2016, Kronenberg, Merkel et al. 2019)*.* In both primary microglia (Calvo,

Zhu et al. 2010, Kronenberg, Merkel et al. 2019) and cell lines (Dimayuga,

Ding et al. 2003, Mencel, Nash et al. 2013, Simmons, Surles-Zeigler et al.

2016), NRG-1 has been documented to exert anti-inflammatory properties

when delivered within a pro-inflammatory context. These anti-inflammatory

properties include decreased release of pro-inflammatory mediators nitric

oxide and superoxides (Dimayuga, Ding et al. 2003), and reduced production

and release of pro-inflammatory cytokines IL-6 and TNF-α (Simmons, Surles-

Zeigler et al. 2016), which perpetuate inflammatory signaling cascades. In N9

mouse microglial cells activated with pro-inflammatory stimulus LPS, NRG-1

alters the regulation of the major inflammatory pathway NF-κB, attenuating

the canonical NF-κB activation of the pathway by inhibiting LPS-induced

phosphorylation and degradation of IκB-α as well as reducing nuclear

translocation of p65 (Simmons, Surles-Zeigler et al. 2016).

PROPOSED WORK

While previous work has confirmed that NRG-1 can alter the inflammatory state of microglia, there has not yet been an extensive study on how NRG-1 alters gene expression of immune cells in inflammation. In this work I will explore gene expression changes influenced by NRG-1 microglia stimulated with pro-inflammatory LPS through the following specific aims.

SPECIFIC AIMS

Specific Aim 1: Establish an *in vitro* culture model of SIM-A9 microglial inflammatory activation to examine the immunoregulatory roles for NRG-1. The work contained in Specific Aim 1 is recorded in Chapter 2 and consists of two major sub-Aims.

Sub-Aim 1A: A key validation will be establishing that SIM-A9 cells express the ErbB4 receptor *in vitro*. NRG-1 mediates its cellular signaling through biding to the ErbB family of receptors. NRG-1 binds preferentially to ErbB3 and ErbB4, but as only ErbB4 contains an active intracellular domain, responsiveness to NRG-1 treatment is determined by ErbB4 expression. Also, ErbB4 has been shown to be the primary NRG-1 receptor expressed in microglia. Therefore, we will examine ErbB4 expression in the SIM-A9 model.

Sub-Aim 1B: Establish experimental protocols to induce a range of inflammatory activation states in SIM-A9 microglia in order to identify changes in inflammation in response to NRG-1 administration. This includes preliminary work to determine proper doses and experimental timing for

optimal response. Inflammatory activation will be achieved and modified through stimulation with well-characterized pro-inflammatory and anti-inflammatory activators, LPS and IL-4 respectively. The effects of NRG-1 stimulation will be explored in comparison to LPS and in comparison to changes achieved when IL-4 and LPS are applied concurrently. Inflammatory activation state will be classified and defined in terms of expression of well-known inflammatory markers. These including production of the pro-inflammatory markers nitric oxide (NO) and Interleukin-1β (IL-1β), anti-inflammatory markers arginase 1 (Arg1) and interleukin 10 (IL-10), and activation marker interleukin 6 (IL-6).

Specific Aim 2: Define the gene expression changes caused by NRG-1 to the SIM-A9 microglial inflammatory state. We will induce inflammation with pro-inflammatory LPS. Stimulation with LPS and anti-inflammatory IL-4 will be used as a benchmark.

Sub-Aim 2A: Evaluate the genetic response to NRG-1 exposure using the gene expression levels of a NanoString panel of 285 inflammatory genes. Gene expression will be defined from mRNA count. Counts of mRNA will be used to determine differential gene expression. We will determine how NRG-1 changes inflammation by comparing to LPS-induced gene expression, while using changes achieved by concurrent stimulation with LPS and IL-4 as a reference for how an anti-inflammatory factor changes on LPS-induced gene expression. These results are shown and discussed in Chapter 3.

<u>Sub-Aim 2B</u>: Identify and analyze the unique gene expression changes of NRG-1 stimulation by expanding the scope explored genes from inflammation to 757 additional genes that are important in neuropathology and include non-inflammatory systems. Using a larger panel of gene expression probes gives a more complex and nuanced pictures of microglial inflammatory activation. Work from Sub-Aim 2B is presented in Chapter 4. Table 1-1 outlines NanoString Panels used in Aim 2.

Table 1-1
General Information About NanoString Panels

	Inflammation	Neuroinflammation
# Genes	248	757
Themes (# genes per theme)	Inflammation	Inflammation Cellular Stress Neurons and Neuropathology

CHAPTER 2 AIM 1- EVALUATING THE EFFECTS OF NRG-1 ON ESTABLISHED BIOMARKERS OF INFLAMMATION

ABSTRACT

This chapter contains all the work and preliminary experimental results to carry out Aim 1 of this work. For these experiments it was necessary to move from a sequential model of exposure to inflammation modulating molecules to a simultaneous model of application. We utilized the SIM-A9 microglial cell line and chose to stimulate with the very well-characterized inflammation paradigm using pro-inflammatory molecule lipopolysaccharide (LPS) as a pro-inflammatory stimulus and IL-4 as an anti-inflammatory stimulus. We tested doses of stimulatory molecule, the time-course of released factors upon stimulation, and the effects of different treatment protocols.

Cell lines can differ in their response to certain stimuli, so it is necessary to test experimental conditions and find those in which the cells respond maximally to the chemical exposures of interest. In this work, we tested various doses of pro- and anti- inflammatory factors, the time-course of released factors upon stimulation, and the effects of different treatment protocols in a SIM-A9 mouse microglial cell line. We looked at anti-inflammatory stimulus IL-4 over a range of concentrations spanning at least an order of magnitude (two orders of magnitude measured by concentration, one measured by molarity) and found measurable reduction of inflammation as measured by nitric oxide production. Finally, we evaluated a range of

NRG-1 doses and established that the therapeutic dose of NRG-1 within this *in vitro* system fell within the range of 10-100 ng/mL.

RATIONALE

The objective of this preliminary work is to establish cell culture protocols for using SIM-A9 microglial cells as an in vitro model to study the ways NRG-1 exposure influences changes in inflammatory phenotype of immune cells. Inflammatory activation studies use a simple protocol of exposing unstimulated cells to activating stimuli individually. However, a more relevant model of pathology and subsequent exposure to NRG-1 as a treatment requires exposing cells to multiple, competing inflammatory stimuli simultaneously. We propose comparing cellular responses elicited from individual pro-inflammatory activating stimulus with LPS to cellular responses measured following combined exposure to NRG-1 and pro-inflammatory activation with LPS to isolate NRG-1 induced changes to inflammation. A similar protocol using anti-inflammatory activation stimulus IL-4 in combination with LPS rather than NRG-1 provides a benchmark of what inflammatory changes can be induced by an anti-inflammatory mechanism. In Specific Aim 1, the objective is to confirm that SIM-A9 cells are an appropriate model in which to study NRG-1 influences on inflammation. This objective will be achieved in two parts. One is to confirm that SIM-A9 cells are responsive to NRG-1 signaling. The second is to establish cell culture conditions that induce measurable inflammatory responses and NRG-1-

induced alterations in those responses in SIM-A9 cells as quantified by traditional inflammatory markers.

OUTLINE OF PROPOSED EXPERIMENTAL SYSTEM
Cell Line

In this work microglial interactions are modeled *in vitro* utilizing spontaneously immortalized SIM-A9 microglial cells (Nagamoto-Combs, Kulas et al. 2014). Cell lines provide a clonal population which can serve as a defined starting point (or "M0") for experiments, allowing the assumption that any changes in microglial phenotype are the result of interactions with applied excitation factors rather than pre-existing heterogeneity. In addition, cell lines allow for further experimentation into the mechanisms of microglial state-change dynamics because cells can be efficiently cultured to provide high yields necessary for performing genetic manipulations (Rodhe 2013, Timmerman, Burm et al. 2018).

Previously published studies into the effects of NRG-1 on microglia *in vitro* from our lab and others have used several cell lines including N9 (Dimayuga, Ding et al. 2003, Simmons, Surles-Zeigler et al. 2016) and BV2 (Mencel, Nash et al. 2013). We have decided to change to the SIM-A9 microglial line, because while a recent addition to the microglial field, SIM-A9 cells offer several advantages over previously used cell types. The SIM-A9 cell line was derived from primary microglia isolated from mixed glial culture of neonatal murine cerebral cortex through spontaneous immortalization rather than viral

transformation, genetic manipulation, chemical influences, or radiation (Nagamoto-Combs, Kulas et al. 2014). Spontaneous immortalization likely offers improved reliability of data as there have been literature reports that induced cell lines have problems with long-term retention of primary microglial properties(Timmerman, Burm et al. 2018). In addition, the genome of this cell line has not undergone the specific alterations from its primary source that are inevitable in cell lines immortalized by other means. In contrast, immortalization of N9 and BV2 cell lines was artificially induced through viral transfection of the v-myc or v-myc and v-raf oncogenes, respectively. SIM-A9 cells do not rely on the addition of growth factors or the support of feeder cells, which simplifies culture protocols and limits sources of inter-experiment variability. This cell line is uniquely suited to the proposed research for these reasons and because it is the only commercially available microglial cell line sourced from the cortex.

Defining Pro-Inflammatory and Anti-Inflammatory Outcome Measures

SIM-A9 cells were created in 2014 and have been utilized in few published studies (Nagamoto-Combs, Kulas et al. 2014, Farrell, Borazjani et al. 2016, Zhang, Wang et al. 2018, Bonaterra, Mierau et al. 2020, Dave, Ali et al. 2020, Sibbitts and Culbertson 2020). To determine proper doses of my various induction factors, I must track pro and anti-inflammatory character of my cultured cells. However, the terms pro-inflammatory and anti-inflammatory are vague and broadly defined, encompassing many cellular behaviors and

functions which can be identified in many ways. Tracking dose-wise effects of each of stimuli required well characterized, specific markers of immunological states which could be assayed simply and reliably. Adequately identifying microglial inflammatory state will require use of both pro-inflammatory and anti-inflammatory markers.

In vitro markers of pro-inflammatory microglial activation include production and release of pro-inflammatory cytokines or chemokines, production of cytotoxic factors such as reactive oxygen species (ROS), induction of factors that alter local tissue permeability such as nitric oxide (NO) and the induction of pro-inflammatory gene expression. The canonical pro-inflammatory cytokines, which are routinely documented to be elevated in neuroinflammatory diseases and stroke, as well as specifically induced by pro-inflammatory stimulus with LPS include, IL-1β, TNF-α, and IL-6. In addition, NO is a common biomarker used to confirm proinflammatory LPS stimulation *in vitro* (Nagamoto-Combs, Kulas et al. 2014, Shahriary, Kataria et al. 2019). NO production can be quantified quickly, cheaply and in a high throughput manner using the Griess reagent. In this work we chose to use NO production as the outcome measure to determine effective dosing in all our stimulation conditions. Released IL-6 protein can be measured through ELISA assay of cell culture supernatant and was used as an additional confirmation of pro-inflammatory activation. Finally, rt-qPCR was used as an additional genetic confirmation of proteomic results. We used validated

transcripts for pro-inflammatory cytokines IL-1β and IL-6 to confirm pro-

inflammatory activation.

The canonical biomarker of IL-4 anti-inflammatory activation is production of

Arginase 1. An additional common biomarker for anti-inflammatory state is

the expression of macrophage mannose receptor (MmR, or CD206) on the

cell surface. To identify anti-inflammatory activation, we detected Arg1

expression through western blotting. As a confirmation of proteomic results,

we used validated transcript to identify MmR expression.

MATERIALS AND METHODS
Chemicals

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
ErbB4	Santa Cruz	Sc-283
Carboxy-terminal epitope ErbB4 (HFR1)	Invitrogen	13-9687-82
Arginase 1	Santa Cruz	Sc-271430
Chemicals, peptides, and recombinant proteins		
Interleukin-4 (IL-4) mouse, recombinant; expressed in E. coli	Sigma-Aldrich	Cat#SRP3211, Lot#0309AFC49
Lipopolysaccharide (LPS)	Millipore Sigma Calbiochem	Cat# 437627-5mg Lot# 2780263
Recombinant human neuregulin1-beta1 EGF-like domain (NRG), R&D systems. Reconstituted in 1% BSA in PBS	R&D Systems	Cat# 396-HB Lot# ACD1617121

CELL CULTURE

The murine SIM-A9 microglial cell line was purchased through ATCC. SIM-

A9 stock cells were cultured in a 1:1 ratio of Dulbecco's modified Eagle's

media and Ham's F-12 nutrient mixture (DMEM/F12) with 2mM glutamate

and HEPES buffer (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA,

USA) supplemented with 10% fetal bovine serum (FBS, Corning), 5% horse

serum (HS, Corning), streptomycin (100 U/mL) and penicillin (100 U/mL) (ATCC). Cells were maintained in 95% air, 5% CO_2-humidified atmosphere at 37°C and grown in T75 flasks monitored daily and passaged non-enzymatically with a solution of ice-cold PBS containing 1mM EDTA + 1 mM EGTA +1mg/mL glucose when cell monolayer reached 75-85% confluency. For experiments using various factors to induce immunological state changes (induction factors), cells were plated into either 12- or 24-well plates at a density of 100K cells/cm^2. For all 24-hour exposures, cells were plated in serum-free media containing induction factors at the final concentrations indicated. For experiments involving shorter exposures, cells were seeded in serum free (SF) media and allowed to attach overnight prior to total media change and application of media containing induction factors.

MEASUREMENT OF NITRIC OXIDE PRODUCTION

NO present in cell culture supernatants was quantified using the Griess reaction (Promega, Madison, WI, USA) according to manufacturer's instructions for the microplate assay. Briefly, micro-plate assay required 50 µl of cell culture supernatant to be added to wells of 96 well plate. 50 µl sulfide solution is added for 5 minutes followed by 50 µl NED solution, after which plate is allowed to react protected from light for 5-10 minute as color develops. Plates are read within 30 minutes of the final incubation.

PROTEIN ANALYSIS METHODS

Enzyme-linked Immunosorbent Assay (ELISA)

Cell culture supernatants were collected, centrifuged at 1000 g to remove

cellular debris and stored at -80° C until assayed. We assayed for IL-6 with

the Quantikine ELISA (R&D Systems, Minneapolis MN, USA) in microplates

as per manufacturer's instructions and read plates using Varioskan LUX

multimode microplate reader (Thermo Fisher Scientific, Inc) plate-reader or

GloMax plus multi-detection system (Promega, Madison, WI, USA).

Protein Isolation for Western Blotting

Protein was harvested by direct lysis of cells in the culture dish using M-PER

(Pierce Biotechnology, Rockford, IL) or RIPA (ThermoFischer) lysis buffer

containing protease and phosphatase inhibitors for isolation of cytosolic or

membrane-bound proteins, respectively, according to the manufacturer's

instructions. Protein concentrations were determined via Braford protein

assay (Bio-Rad Hercules, CA, USA) and a minimum of 15 ug total protein

lysates per well were loaded into stain free PROTEAN TGX gels (Bio-Rad)

for protein separation via sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE). Gels were blotted onto polyvinylidene

difluoride (PVDF) membranes using Bio-Rad Trans-turbo system (Bio-Rad).

Membranes were blocked in with 3% BSA or 10% milk diluent (Bio-Rad) in

0.1% Tween-20/Tris-buffered saline (TBST) for 1 hour at room temperature

and subsequently incubated in primary anti-body diluted in TBST + 5% milk

diluent overnight at 4°C. The following day, blots were washed and incubated

in horseradish peroxidase-conjugated secondary against host of primary in

TBST + 5% milk + streptacin (Bio-Rad) for 1 hour at room temperature and

finally developed in clarity ECL (Bio-Rad). Images were taken on the

Chemidoc system and analyzed in image lab software (Bio-Rad). Bands

were normalized using total protein content per lane as determined by stain-

free image and normalization was confirmed using GAPDH image.

RESULTS AND DISCUSSION
SIM-A9 Cells Express Consistent Level of ErbB4 Receptor Under Naïve and Pro-Inflammatory Stimulation

In order to establish SIM-A9 cells as an appropriate model to study NRG-1

signaling it was necessary to show that SIM-A9 cells express receptors to

detect NRG-1. The main NRG-1 receptors are ErbB3 and ErbB4, but only

ErbB4 is able to signal thorough homodimerization, making ErbB4

expression necessary and sufficient for competent NRG-1 signaling. NRG-1

binding to ErbB4 triggers dimerization and activation of intracellular signaling

transduced via phosphorylation events at the intracellular tyrosine kinase

domains on receptors. In addition to the trans-membrane signaling, ligand

binding of ErbB4 can also initiate cleavage of the receptor, generating a

soluble intracellular domain that can go to the nucleus and directly regulate

transcription (Sundvall, Peri et al. 2007). Lysis of the full 180 kDa receptor at

the extracellular juxtamembrane domain results in formation of a 120 kDa

ectodomain that is shed and an 80kDa membrane-bound domain. The 80 kDa domain contains the carboxy-terminal end of the receptor and may stay bound to the membrane or upon cleavage by γ-secretase may become an intracellular domain (ICD) and travel to the nucleus. Western blotting confirms that SIM-A9 cells constitutively express ErbB4 (Figure 2-1). Furthermore, pro-inflammatory activation via LPS did not alter ErbB4 expression over a range of 2.5 ng/mL to 10μg/mL, which represented the highest and lowest LPS concentrations used for *in vitro* stimulation of cell cultures in literature. It has previously been reported that the effects of NRG-1 are seen most strongly in cells exposed to inflammatory stimulation, so the consistent expression between various states of pro-inflammatory stimulation suggests that the increase in NRG-1 effect is not likely due to an increase in overall number of receptors.
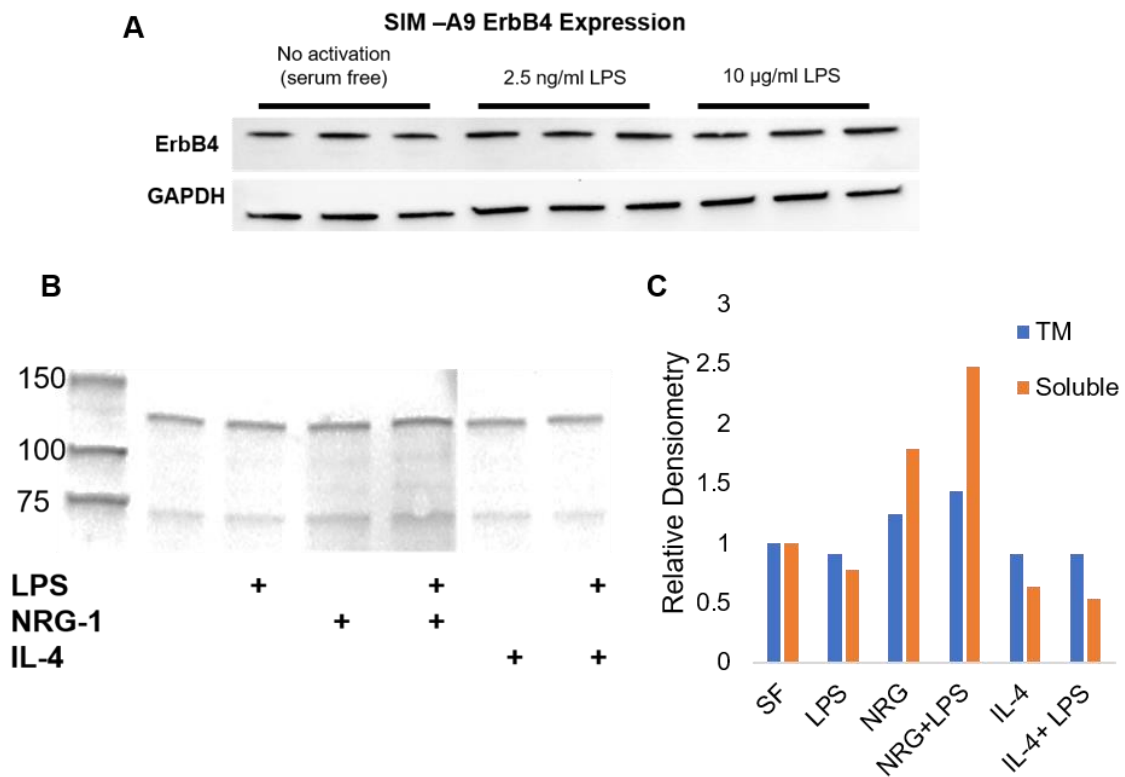
Figure 2-1: ErbB4 Expression in SIM-A9 Cells after 24-hour exposure to various treatments

A) Western blot shows consistent ErbB4 levels over a range of inflammatory stimulation from none (SF) to high levels of LPS (10μg/mL). Cells were plated under serum-free conditions in media containing the treatments indicated and collected after 24 hours of exposure to treatment for a total of 24 hours in culture. Antibodies: ErbB4 sc-283; GAPDH HRP-60004 **B)** Western blot and **C)** quantification shows level of full-length (180 kDa) and cleaved (80 kDa) ErbB4 in SIM-A9 cells exposed to a range of inflammatory molecules. Lane 1: Serum Free (baseline) Lane 2: 1 μg/mL LPS
Lane 3: 100 ng/mL NRG-1 Lane 4: 1 μg/mL LPS + 100 ng/mL NRG-1
Lane 5: 50 ng/mL IL-4 Lane 6: 1 μg/mL LPS + 50 ng/mL IL-4. SIM-A9 cells plated in serum free media and starved for 24 hours followed by full media change to serum-free media supplemented with indicated molecules. Cell lysates collected after 24 hours exposure to stimuli; total time in culture 48 hours. Normalization by total protein content using stain-free gels (Bio-Rad). Antibodies: Carboxy-terminal epitope of ErbB4 (HFR1) 13-9687-82.

To gain a more nuanced understanding of how our proposed treatments might affect ErbB4 expression and trafficking, we utilized an antibody detecting the carboxy-terminal end of the receptor and were able to detect ErbB4 cleavage by the molecular weight of the bands. Cells were plated in serum-free medium and starved for 24 hours before 24-hour exposure to LPS, IL-4, NRG-1 or a combination. All conditions showed approximately equivalent levels of the full-length receptor, although NRG-1 exposure did slightly elevate receptor expression. NRG-1 exposure led an increase in cleavage fragment. Anti-inflammatory IL-4 appeared to reduce ErbB4 cleavage while pro-inflammatory LPS on its own caused minimal change or in conjunction with IL-4, but did further increase cleavage when delivered with NRG-1. Approximate parity between baseline levels of the full-length transcript and both pro-inflammatory and anti-inflammatory treatments indicates that LPS and IL-4 have little effect on generation of ErbB4. Strong reduction of 80 kDa fragment in IL-4 treatments may indicate an antagonistic relationship between anti-inflammatory environments and ErbB4 cleavage which was not observed with pro-inflammatory treatments. Finally, the increase in full-length transcript on NRG-1 conditions suggests cells do not become sensitized to NRG-1/ErbB4 signaling over the 24-hour exposure, while increased membrane-bound fragment confirms that NRG-1 is activating ErbB4 receptors, and the activation or cleavage may be further increased by pro-inflammatory environments.

PRO-INFLAMMATORY EXCITATION WITH LPS

The range of LPS concentration used to stimulate microglia *in vitro* has been noted to vary widely from the nanogram/mL range to microgram/mL concentrations (Chhor, Le Charpentier et al. 2013). Previous studies in our lab stimulated N9 microglia with 10μg/mL, while LPS concentrations used to stimulate SIM-A9 cells within published literature varied from 2.5 ng/mL (Nagamoto-Combs, Kulas et al. 2014, Farrell, Borazjani et al. 2016) to 25 μg/mL (Bonaterra, Mierau et al. 2020).

To determine the optimal dosage to produce a pro-inflammatory response to LPS stimulation in SIM-A9 cells *in vitro,* we tested a range of concentrations from 1ng/mL to 10μg/mL. Indicators of pro-inflammatory response were release of inflammatory activation marker IL-6 and generation of NO. SIM-A9 cells were plated in serum-free condition and serum-starved for 12 hours to allow attachment to plates, and then exposed to LPS at various concentration for 24 hours. Following 24-hour exposure, media was collected and measures. We saw a dose-wise increase in both inflammatory markers over the range of LPS concentrations (Figure 2-2). Specifically, IL-6 plateaued around 100ng/mL while NO showed a steady increase over all doses. To minimize material usage, we looked for the smallest concentration that was significantly different in both markers which corresponded to 1ug/mL. Therefore, we determined that 1μg/mL LPS was the ideal dose to illicit a

strong pro-inflammatory response in SIM-A9 cells and used this

concentration for LPS stimulus in all experiments going forward.

LPS Dosing

IL-6 Production In Response to LPS Exposure Over a Range of Concentrations

	PBS	SF	1 ng/mL	10 ng/mL	100 ng/mL	1 µg/mL	2.5 µg/mL	10 µg/mL
Mean (pg/ml)	23.5	37.8	988.9	1951.7	2228.7	2323.4	2458.3	2416.5

LPS Concentration (g/mL)

Nitrite Production In Response to LPS Exposure Over a Range of Concentrations

	SF	PBS	1 ng /mL	10 ng /mL	100 ng /mL	1 µg/mL	2.5 µg/mL	10 µg/mL
Avg	0	0	1.2	2.5	3.6	4.3	4.6	5.8

Figure 2-2: LPS Produces a dose-wise increase in pro-inflammatory markers

A) Levels of IL-6 in culture supernatants were measured using an ELISA. Data shown as averages of n=3. B) NO production in cell culture supernatants was measured using the Griess reagent. These are markers used to characterize and identify a pro-inflammatory activation state in microglia and macrophages. IL- 6 is a marker of inflammatory activation and NO is produced via NOS2 in response to a pro-inflammatory insult.

ANTI-INFLAMMATORY ACTIVATION WITH IL-4

IL-4 Dosing

Table 2-1
Range of IL-4 Doses Evaluated

Concentration	Molarity
2.5 ng/mL	0.185 nM
5 ng/mL	0.37 nM
10 ng/mL	0.74 nM
25 ng/mL	1.85 nM
50 ng/mL	3.7 nM
100 ng/mL	7.4 nM

Cells were plated in SF media at 100K cells/cm^2 and allowed to attach overnight. After 12 hours in culture, media containing IL-4 at final concentrations listed in Table 2-1was applied. After 24 hours of exposure to IL-4 containing media, supernatants were collected, and cells lysed to collect protein samples for western blotting as outlined in sections above. Anti-inflammatory effects of IL-4 treatment were clearly shown at all doses of IL-4 used in our experiments (Figure 2-3). IL-4 reduced LPS-induced NO production by roughly half over an exposure period of 24 hours. Notably, increased IL-4 dose did not result in significant changes in NO reduction over the range from 10ng/mL to 100 ng/mL. However, a strong dose wise increase was observed in expression of the anti-inflammatory marker Arg1. Over the range of IL-4 doses we tested, Arg1 showed a monotonic increase with no clear plateauing effect. IL-4 doses above 25ng/mL showed both robust expression of anti-inflammatory Arg1 and significant reduction in pro-inflammatory NO (Figure 2-4). For subsequent experiments we used 50ng/mL IL-4 as our optimized chemical induction of anti-inflammatory state in SIM-A9 microglia.

NO Production Over Range of IL-4 Doses
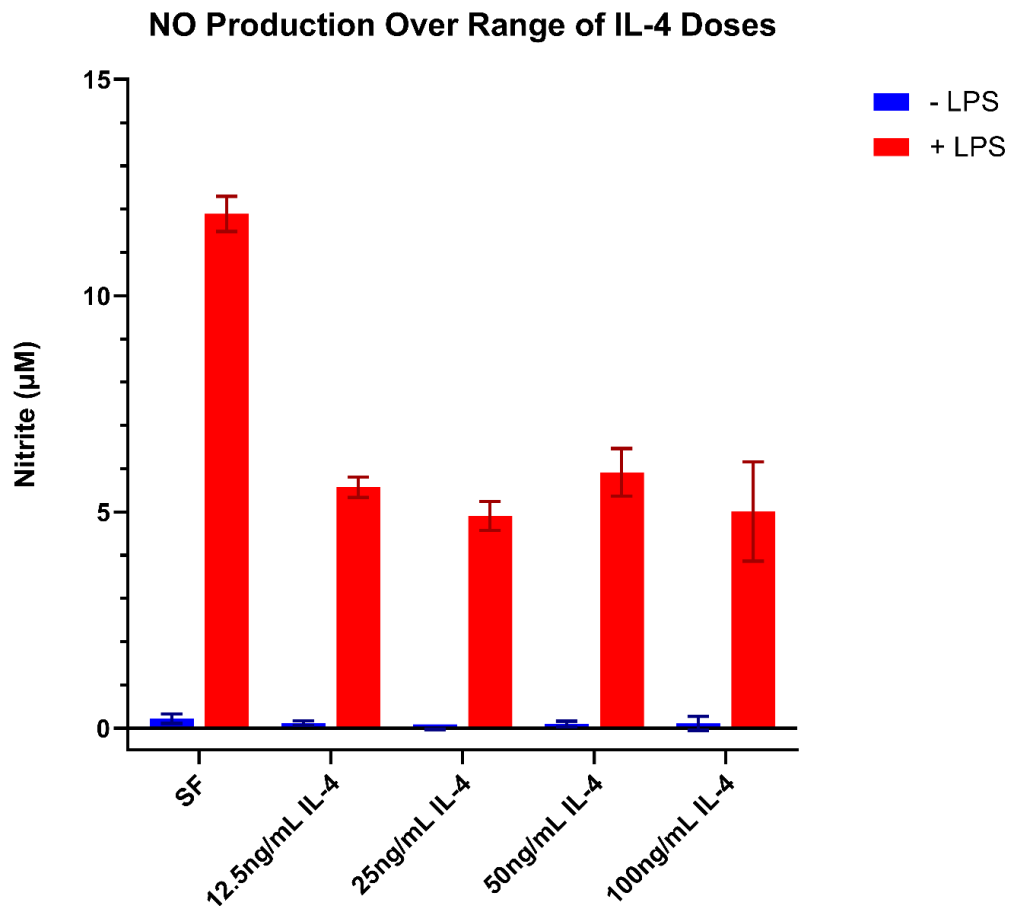
Figure 2-3: Nitric oxide production in SIM-A9 cells as a function of IL-4 dose in the presence and absence of LPS

SIM-A9 cells were exposed to a range of IL-4 concentrations in the presence or absence of LPS for 24 hours prior to collection of samples. Supernatants were collected and assayed for NO production using the Griess reagent and quantified via spectrophotometry. Bars display mean ± standard deviation for 2 independent runs of the experiment, each time n=3.
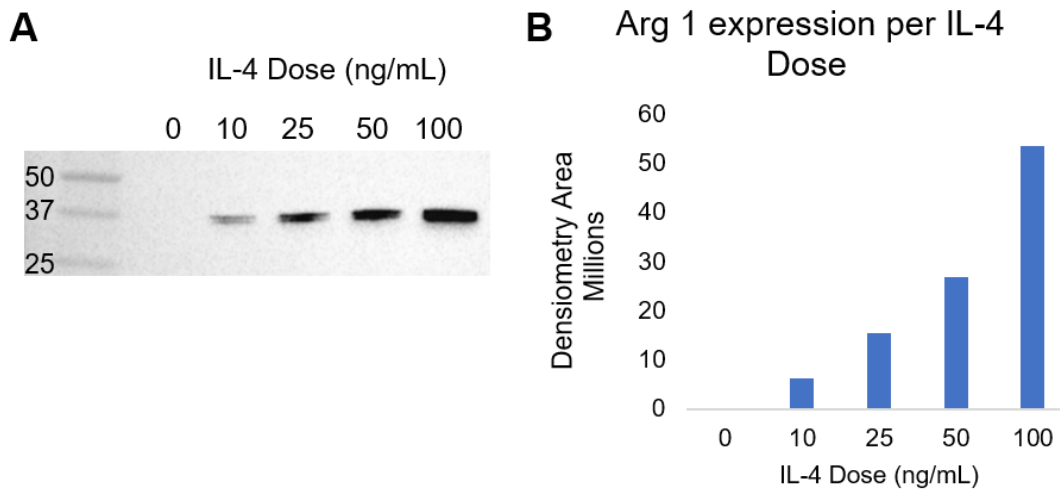
Figure 2-4: IL-4 produces a dose-wise increase in Arginase 1 expression
SIM-A9 cells were exposed to varying doses of IL-4 from 10ng/mL to 100ng/mL for 24 hours. Protein was collected after 24 hours exposure (total of 48 hours in culture). Arginase 1 (Arg1) expression was evaluated via western blot **(A)** and quantified (**B**) by densitometry determined using Imagelab software to analyze ECL images taken on chemidoc. Antibodies: Arg1 sc-271430

EMPIRICIAL ASSESSMENT OF TIMING FOR STIMULATION SUDIES

In the first experiment, cells were allowed to attach in SF media overnight,

and then experimental media was added and results collected after 3, 6, and

24 hours. As in LPS dosing experiments, IL-6 and NO production were used

to identify pro-inflammatory character. LPS induced robust and significant

increases in NO and IL-6 after 24 hours of exposure (Figure 2-5).

Unstimulated cells in serum free media did not produce significant amounts

of pro-inflammatory markers NO or IL-6 at any of the time points studied. At

the 6 hour and 24 hour timepoints LPS induced an increase in NO production

IL-6 release, but the NO increase was not statistically significant at 6 hours.

Therefore, we concluded that an exposure of 24 hours was optimal to

achieve maximal measurable inflammatory response and able to induce a

measurable inflammatory state in SIM-A9 microglia. An exposure time of 24

hours was used to establish inflammatory state in all subsequent

experiments.
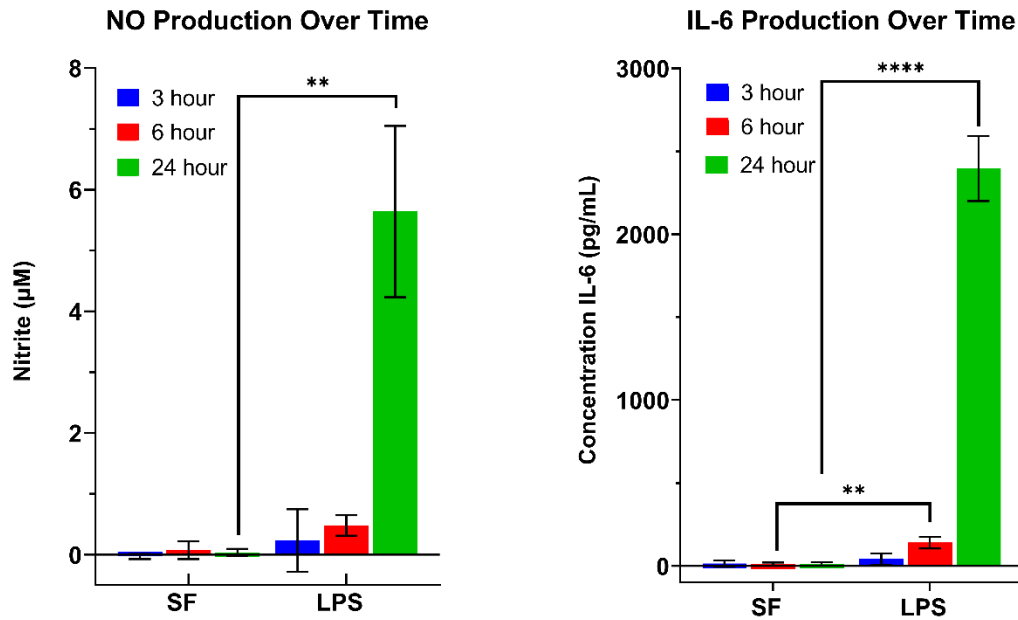
Figure 2-5 Time-course of LPS-induced inflammatory markers

Measurable response of SIM-A9 cells to LPS dosing at 1μg/mL evaluated at 3, 6, and
24 h. While 6 hours shows significant difference relative to unstimulated cells in serum
free media (SF) for IL-6 production, only the 24 h timepoint showed significant difference
in both NO and IL-6 production. Based on this result, the 24 hour timepoint selected as
the reference time to perform gene expression analysis. Nitric oxide measured with
Griess reagent, IL-6 production measured by ELISA and measured on the Victor II
spectrophotometer at 450 nm.

NRG-1 DOSING

Evaluating the Direct Effects of NRG-1 on Microglia

To determine effective dose of NRG-1 to stimulate response in SIM-A9

microglia we evaluated reduction in LPS-induced NO production over a range

of NRG-1 concentrations from 50 ng/mL to 1 µg/mL. Doses covered are

shown in Table 2-2.

Table 2-2
Range of NRG-1 Doses Evaluated

Concentration	Molarity
50 ng/mL	6.25 nM
100 ng/mL	12.5 nM
250 ng/mL	31.25 nM
500 ng/mL	62.5 nM
750 ng/mL	93.75 nM
1000 ng/mL (1 µg/mL)	125 nM

Cells were plated in SF media and allowed to attach for 12 hours. The next

day media was changed to media containing varying concentrations of

NRG-1 (shown in Table 2-2) in the presence or absence of 1µg/mL LPS and

collected after 24 hour exposure.
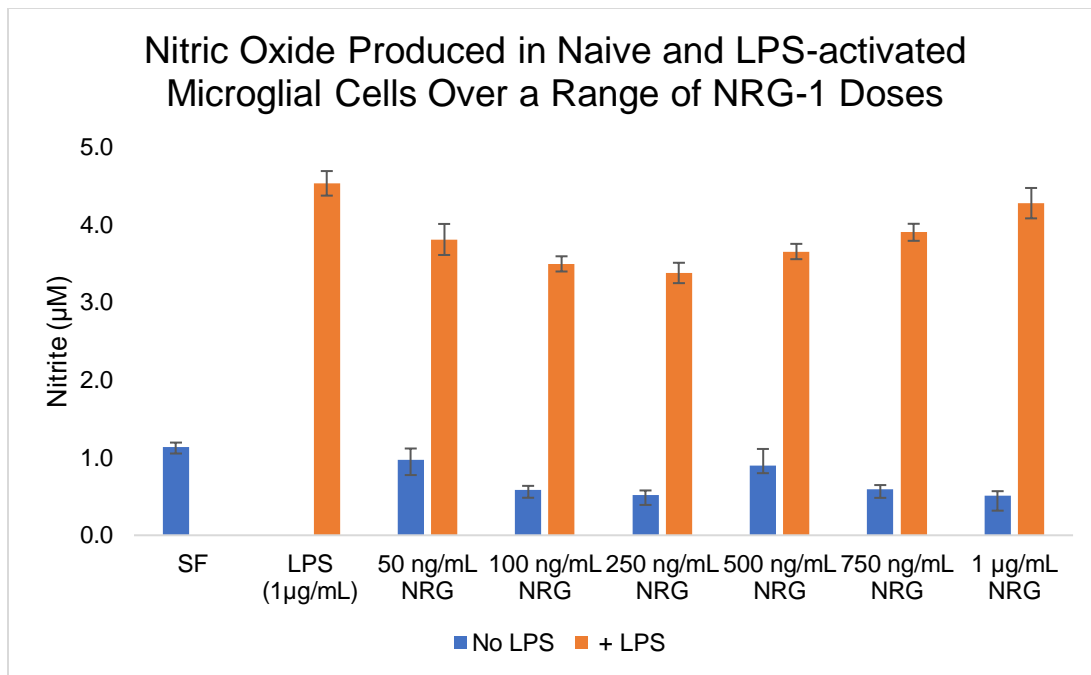
Figure 2-6 Effect of stimulation with NRG-1 on NO production over a range of doses

NRG-1 alone does not induce NO production. When delivered in the presence of LPS, NRG-1 did not significantly reduce NO production relative to LPS stimulation control at any dose over the range of NRG-1 doses assayed. There was a nonsignificant trend towards reduced NO at 100 and 250 ng/mL NRG-1. Bars are representative of 3 repeated runs of this experiment. N = 3 for each bar. Error shown as mean ± SD.

Results indicate that NRG-1 treatment on its own does not significantly induce NO production Figure 2-6. The presence of NRG-1 treatment in conjunction with pro-inflammatory treatment moderately diminished the pro-inflammatory NO production by LPS when compared to LPS alone. There is no clear dose-wise dependence of NO reduction for the range of NRG-1 concentrations used in this study. For lack of clearer metric, we will use 100ng/mL NRG-1 going forward.

In this paradigm, when IL-4 is delivered simultaneously with LPS, the influence of IL-4 significantly reduces the ability of LPS to increase NO

production. Significant dampening of LPS-induced effects indicates that the anti-inflammatory mechanism of IL-4 reverses or prevents pro-inflammatory signaling.

The effect of NRG-1 on LPS-induced NO production was not as pronounced as the reduction achieved by IL-4 (Figure 2-7), which likely means NRG-1 does not work through the same mechanism of strongly reversing or preventing cellular responses to pro-inflammatory stimuli. However, NO is a multifunctional molecule that is necessary for a strong and competent immune response. NO functions as a vasodilator, allowing increased infiltration of immune cells to the area of damage (Steinert, Chernova et al. 2010). Within neuroinflammation the role of NO is even more complex because NO also functions as a neurotransmitter (Steinert, Chernova et al. 2010). NO is essential for neuronal development and survival and regulates synaptic activity and excitability (Steinert, Chernova et al. 2010, Yuste, Tarragon et al. 2015).

However, dysregulation of NO can become dangerous. Pro-inflammatory stimuli such as LPS induce expression of inducible nitric oxide synthase (iNOS or NOS2), which leads to a sharp increase of NO (Murray, Allen et al. 2014, Lively and Schlichter 2018). While overproduction or chronic release of NO can generate cytotoxic reactive nitrogen and reactive oxygen species and contribute to neurodegeneration over time, acute NO production is a necessary part of the immune response (Steinert, Chernova et al. 2010,

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Yuste, Tarragon et al. 2015) Therefore, NO reduction is not necessarily a

desirable outcome within the context of generating a complete and

competent immune response. Our finding that NRG-1 does not show a

strong reduction in NO could indicate that NRG-1 will not interfere with the

necessary parts of the proinflammatory immune response and may be key to

understanding the efficacy of NRG-1 as a treatment.
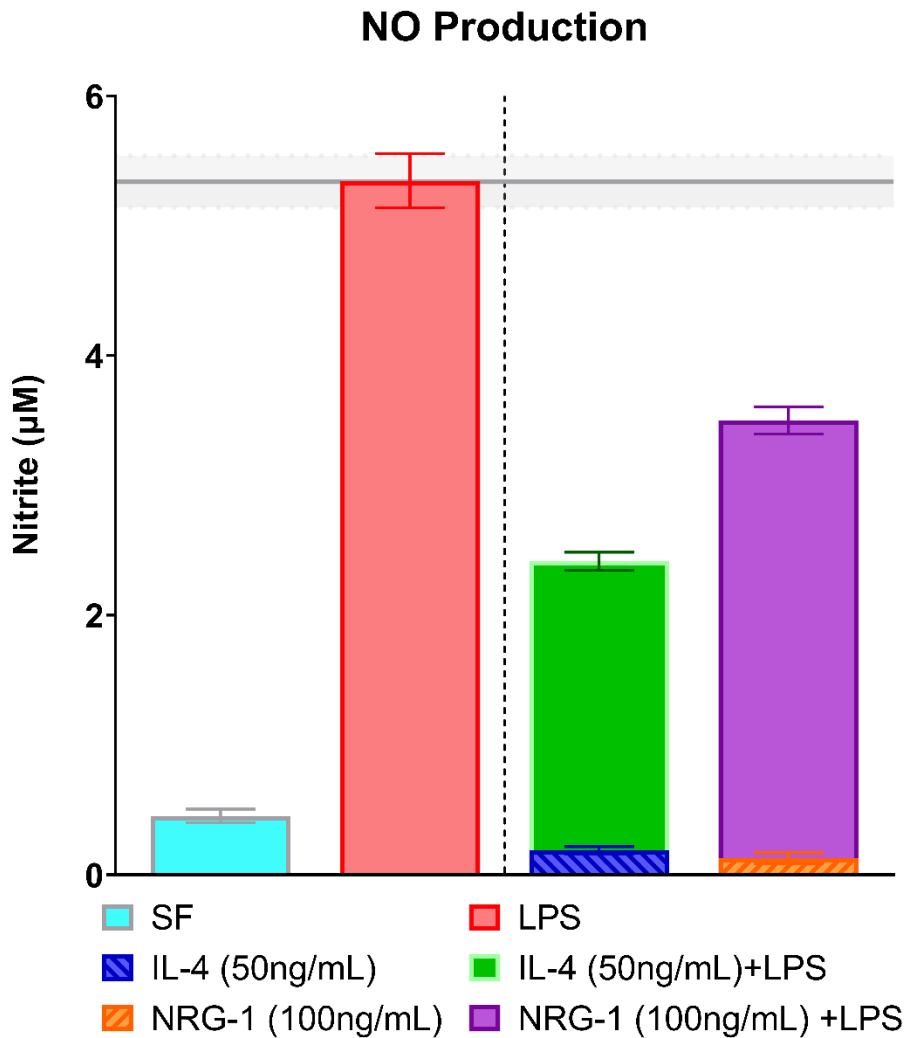
NO Production

Figure 2-7 NO production of NRG-1 dose relative to IL-4

Cells were plated in serum free media for 24 hours then exposed to indicated treatments for 24 hours. Treatment paradigm tested over a range of chemical induction factors. Horizontal grey line indicates LPS-induced NO level. Controls shown to left of dotted line for reference. All bars to the right of the dotted line are shown superimposed with the no LPS control for indicated chemical stimulation. NO reduction by IL-4 is typically understood as an anti-inflammatory outcome. As can be seen, NRG-1 is not able to reduce LPS-induced NO production to the same level as IL-4. NO present in media supernatants measured using Griess reagent and quantified by spectrophotometer absorbance reading. Bars represent mean value ± SD. N=3 for all conditions.

EVALUATION OF IMMUNE STATE INDUCED BY EXPERIMENTAL STIMULATIONS

To confirm the stimulations outlined above were successfully inducing alterations in SIM-A9 inflammatory state, we quantified expression of a few well-known and well-characterized traditional pro-inflammatory and anti-inflammatory markers. These markers were chosen for their ubiquity in the literature and were outlined above in the "Defining Pro-Inflammatory and Anti-Inflammatory Outcome Measures" section.

PRO-INFLAMMATORY MARKERS INCLUDED IL-6 AS MEASURED BY ELISA AND NITRIC OXIDE (NO) QUANTIFIED USING THE GRIESS REAGENT. BOTH OF THESE HAD BEEN USED PREVIOUSLY TO CHARACTERIZE PRO-INFLAMMATORY STATE IN LPS DOSING EXPERIMENTS (SECTION

The range of LPS concentration used to stimulate microglia *in vitro* has been noted to vary widely from the nanogram/mL range to microgram/mL concentrations (Chhor, Le Charpentier et al. 2013). Previous studies in our lab stimulated N9 microglia with 10μg/mL, while LPS concentrations used to stimulate SIM-A9 cells within published literature varied from 2.5 ng/mL (Nagamoto-Combs, Kulas et al. 2014, Farrell, Borazjani et al. 2016) to 25 μg/mL (Bonaterra, Mierau et al. 2020).

To determine the optimal dosage to produce a pro-inflammatory response to LPS stimulation in SIM-A9 cells *in vitro,* we tested a range of concentrations from 1ng/mL to 10μg/mL. Indicators of pro-inflammatory response were release of inflammatory activation marker IL-6 and generation of NO. SIM-A9 cells were plated in serum-free condition and serum-starved for 12 hours to

allow attachment to plates, and then exposed to LPS at various concentration

for 24 hours. Following 24-hour exposure, media was collected and

measures. We saw a dose-wise increase in both inflammatory markers over

the range of LPS concentrations (Figure 2-2). Specifically, IL-6 plateaued

around 100ng/mL while NO showed a steady increase over all doses. To

minimize material usage, we looked for the smallest concentration that was

significantly different in both markers which corresponded to 1ug/mL.

Therefore, we determined that 1μg/mL LPS was the ideal dose to illicit a

strong pro-inflammatory response in SIM-A9 cells and used this

concentration for LPS stimulus in all experiments going forward.

LPS Dosing above, Figure 2-2). Anti-inflammatory marker Arg1 was assessed via western blot. Real-time quantitative PCR (rt-qPCR) served as an orthogonal confirmation of ELISA results for inflammatory activation marker IL-6 and added detection of pro-inflammatory cytokine IL-1β and anti-inflammatory marker macrophage mannose receptor (MmR, aka CD206/Mrc1/CLEC13D).

Stimulation with NRG-1 alone did not generate IL-6 production nor did addition of NRG-1 reduce LPS-induced IL-6 production when both stimuli were delivered together. IL-4 did not reduce IL-6 production when delivered with LPS, but alone IL-4 did not induce significant production of IL-6 according to the ELISA. However, qPCR indicates IL-4 exposure did increase IL-6 mRNA production (Figure 2-10: qPCR Results, which may indicate that IL-4 induced additional production of IL-6, but not necessarily increased IL-6 release from cells. While IL-6 is often referred to as a pro-inflammatory marker in literature, IL-6 has been reported in both pro-inflammatory and anti-inflammatory stimulation. IL-6 appears to be strongly indicative of inflammatory activation, appearing at elevated levels following most neuroinflammatory events.
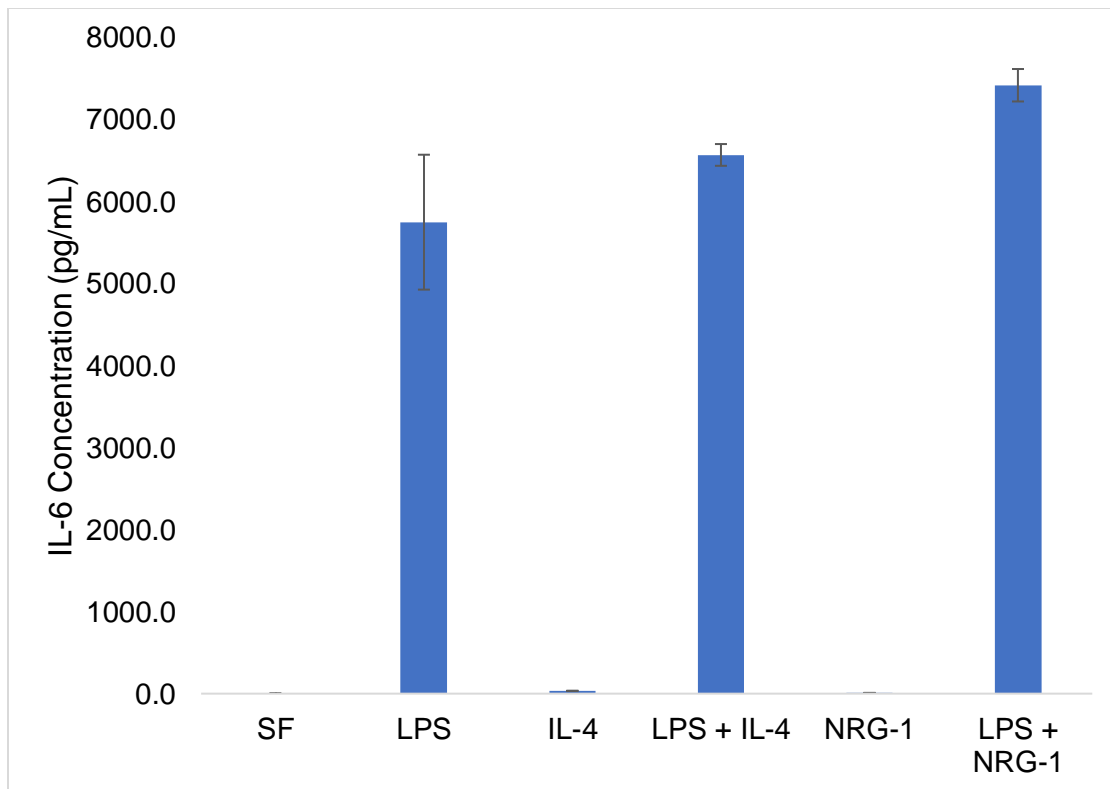
Figure 2-8 IL-6 production over different stimuli conditions
Cell culture supernatants were collected after 24 hours of exposure to indicated factors, alone or concurrent with LPS. Concentrations used for stimulation: LPS =1µg/mL; IL-4 = 50ng/mL; NRG-1 = 100ng/mL. IL-6 production measured by ELISA and reported as mean ± SEM n=3, 2 independent experiments.

The final pro-inflammatory marker used to in these studies was IL-1β (Figure 2-10). This cytokine was strongly induced by LPS but not IL-4 or NRG-1 individually. IL-4 decreased IL-1β expression when delivered concurrently with LPS, which was consistent with literature reporting (Lively and Schlichter 2018) and is in agreement with NO data, indicating that IL-4 achieves anti-inflammatory activation state through a reversal or prevention of LPS-induced expression changes. In contrast, NRG-1 delivered concurrently with LPS did not result in any reduction in IL-1β mRNA expression. This agrees with what we observed for pro-inflammatory marker NO, and again may indicate that

the anti-inflammatory effects of NRG-1 are not likely to be the result of

altering cellular responsiveness to pro-inflammatory stimuli.

Arg1 Expression

| SF | LPS | IL-4 | NRG-1 | LPS + IL-4 | LPS + NRG-1 |
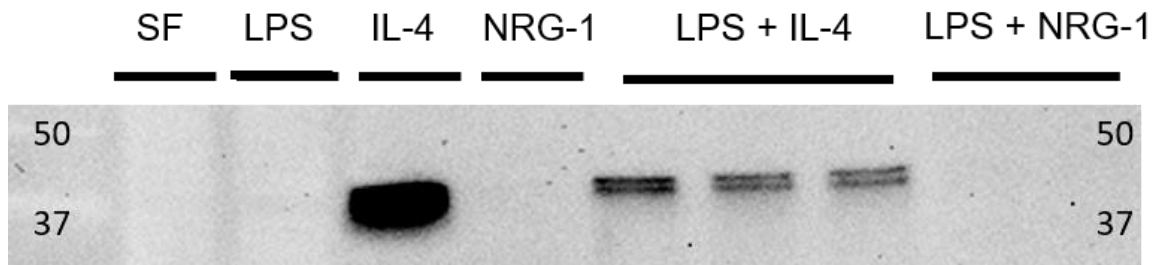
Figure 2-9 NRG-1 treatment does not induce Arginase 1 expression
Protein for western blotting was isolated from cell lysates after 24 hours exposure to indicated treatment conditions. Treatments and Concentrations: SF- serum free media, unstimulated; LPS - 1µg/mL; IL-4 – 50ng/mL; NRG-1 – 100ng/mL, The anti-inflammatory marker Arg1 is only expressed in treatments with IL-4, but notably not within NRG-1 treatments, despite NRG-1 reducing the proinflammatory LPS-induced NO (

NO Production
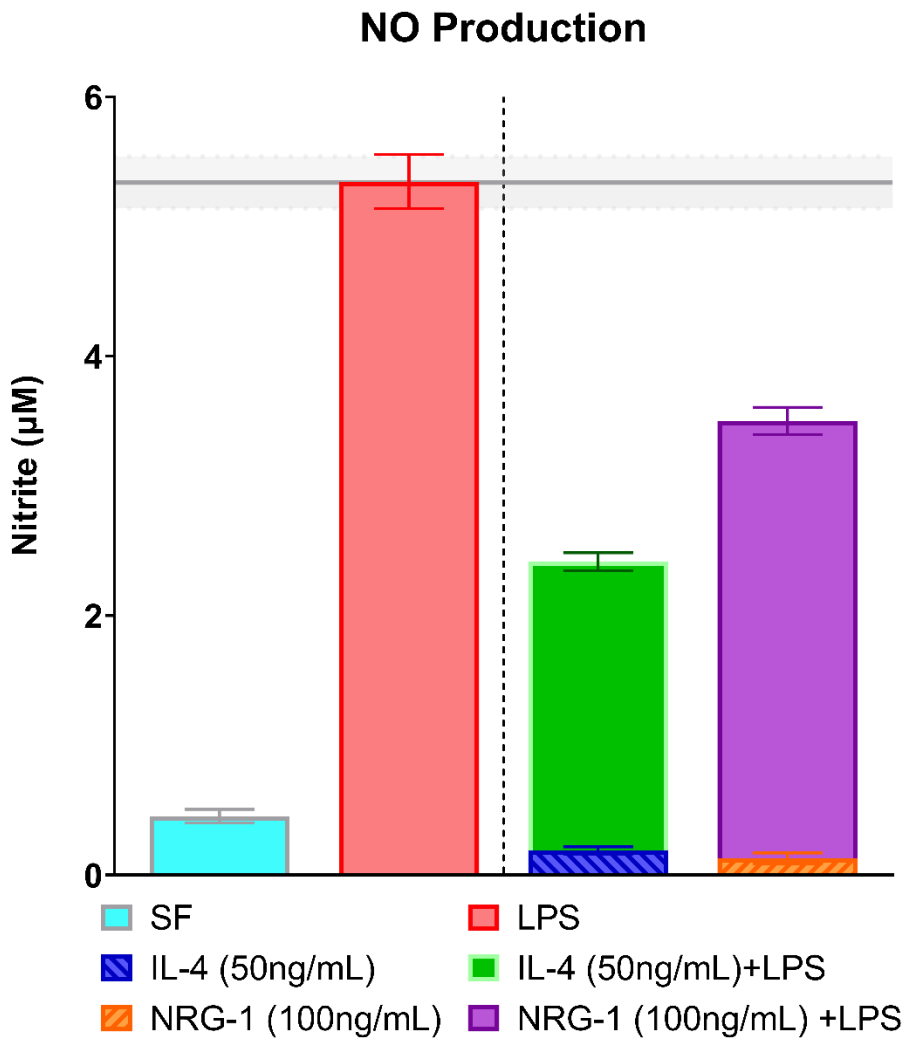
Figure 2-7), indicating that IL-4 and NRG-1 mediate inflammation via differing mechanisms.

Only IL-4 was able to induce expression of anti-inflammatory markers Arg1

and MmR. Arg1 protein was assayed through western blotting (Figure 2-9)

and MmR gene expression quantified using qPCR. Both anti- inflammatory

markers showed similar patterns for expression in the six stimulation

conditions used in these experiments. IL-4 stimulation individually resulted in

significant increase Arg1 and MmR while concurrent treatment with IL-4 and

LPS reduced these levels (Figure 2-9 and Figure 2-10), again indicating an

antagonistic influence of pro-inflammatory LPS stimulation on anti-

inflammatory IL-4 stimulation in which the factors partially reverse or prevent

cell responsiveness to each other.

With the markers used, we defined inflammatory states as follows

LPS – Induced changes include production of IL-6 and IL-1B, NO production

and no production of Arg1 or MmR.

IL-4 induces Arg1 and MmR expression but does not induce IL-6 or IL-1B.

Conjunction with LPS can reduce IL-1B and NO but not IL-6.

NRG-1 reduces NO but not other cytokines assayed. It also does not induce

IL-6 alone.
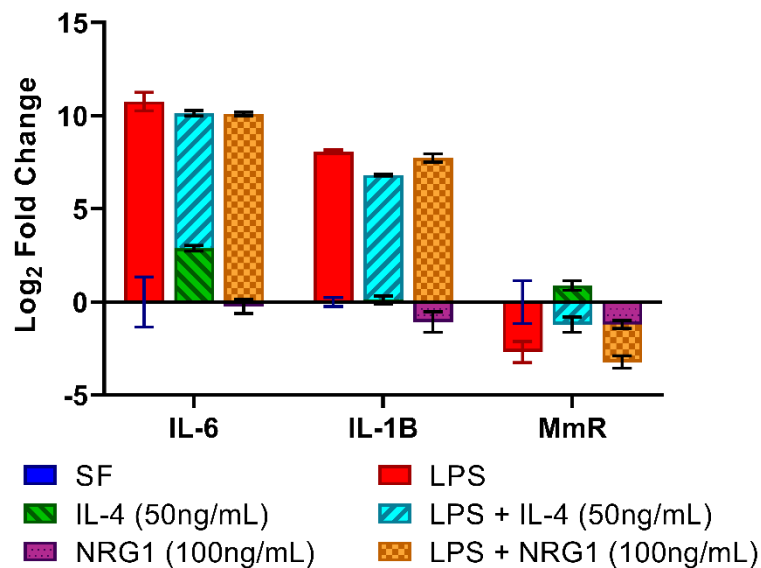
Figure 2-10: qPCR Results
Log2 fold changes in mRNA of IL-6, IL-1β, and MmR as measured through real-time qPCR. Levels known inflammatory state markers were measured for all treatments, individually and in combines treatment with LPS. Results are shown normalized to a baseline of serum free media NRG-1 treatment alone shows minimal change in the immune activation cytokine IL-6, which is increased by both anti-inflammatory and pro-inflammatory stimulation. Pro-inflammatory cytokine IL-1β was increased in pro-inflammatory conditions only and had negligible change in anti-inflammatory stimulation as well as slight decrease under NRG-1 treatment alone. Anti-inflammatory marker MmR was reduced in pro-inflammatory treatments and increased only in anti-inflammatory IL-4 treatment alone.

CONCLUSION OF AIM 1

We began our work by establishing that SIM-A9 microglial cells can be used

as an *in vitro* model for NRG-1 signaling by confirming ErbB4 expression

through western blot analysis.

When delivered alone, NRG-1 did not initiate an increase in any of the pro-

inflammatory markers NO, IL-6, and IL-1β or anti-inflammatory markers Arg1

and MmR. This is in contrast to both LPS and IL-4, each of which was able

to initiate an increase in pro- and anti-inflammatory markers, respectively.

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Previous work led us to hypothesize that NRG-1 treatment would diminish microglial response to pro-inflammatory activation as represented by these markers (Simmons, Surles-Zeigler et al. 2016). While treatment of LPS activated microglia with anti-inflammatory IL-4 was able to consistently show measurable decreases in LPS-induced NO and IL-6 over several time points ranging from 3 to 24 hours, NRG-1 treatment did not reduce IL-6 at any timepoint. Modest reduction of NO was achieved, but was not significant at any dose we measured. These findings led us to conclude that NO production was not a reliable measure of NRG-1 efficacy. In addition, this suggested that the major effects of NRG-1 treatment as seen *in vivo (Xu, Jiang et al. 2004, Xu, Ford et al. 2005)* may not directly affect the regulation of these canonical markers. In addition, NRG-1 did not result in any increase of the anti-inflammatory markers Arg1 and MmR. This may imply that NRG-1 does not operate through traditional anti-inflammatory pathways identified by these canonical anti-inflammatory markers.

These single markers are not sufficient to capture the efficacy and anti-inflammatory effects of NRG-1. Apparently, NRG-1 does not operate as a typical anti-inflammatory treatment, such as IL-4. Therefore, to understand how NRG-1 mediates anti-inflammatory effects, we will expand the scope of markers used to identify changes in the inflammatory state of microglia. High throughput gene expression technologies allow for such expansion. In the

next section we look at a panel of 285 genes associated with inflammation to

attempt to isolate the specific effects of NRG-1 on immune cells.

CHAPTER 3 AIM 2A-ASSESSING THE EFFECT OF NRG-1 ON INFLAMMATORY GENE EXPRESSION IN SIM-A9 MICROGLIA

ABSTRACT

Chapter 2 defined inflammatory state of microglia in terms of a small number of well-known markers. Those markers were useful to define the pattern of inflammatory activation seen in cells that had been stimulated with the known pro-inflammatory LPS and anti-inflammatory IL-4, and could even differentiate the inflammatory state induced by concurrent stimulus with NRG-1 and LPS from LPS alone and LPS + IL-4. However, limiting our discussion to a few markers does not allow for identification of the unique effects of NRG-1 on the inflammatory state of microglia. For this, we must expand the scope of molecules that we are quantifying. In the continuation of this thesis work, we look at the influence of NRG-1 exposure on microglia through the use of gene expression technologies, which can look at hundreds or thousands of genes in a single sample. In this chapter we expand our analysis to make a more thorough analysis of the immune state of the microglia. We use gene expression to identify mechanisms and biomarkers indicative of NRG-1 treatment. In these studies, we utilized the high throughput NanoString technology to expand our characterization to a panel of 248 inflammation genes.

INTRODUCTION

To further understand the unique inflammatory state induced by NRG-1

treatment, this work expands from a few well-characterized markers to a

panel of 248 inflammatory genes.

In this chapter we will use gene expression data identify NRG-1 induced

changes in inflammatory gene expression in SIM-A9 cells. Gene expression

profiles are obtained as mRNA counts using NanoString technology. The

mouse inflammation panel contains 248 genes (Table 3-1; see appendix B).

Samples run on the inflammation panel included 6 exposure conditions with

n=3 for each group (Table 3-2). Samples in each panel were collected from

multiple independent cell culture experiments.

METHODS

Experimental Set-Up and Conditions

For inflammation panel studies, SIM-A9 cells were used a dose of 100ng/mL

NRG-1, consistent with literature and previous studies published in our lab

using N9 cells. To evaluate how NRG-1 is working in the context of

inflammation, we stimulated cultured microglia with LPS either alone or in

conjunction with the IL-4 or NRG-1. Exposure conditions used are listed in

Table 3-1.

Table 3-1
Exposure Condition for Inflammatory Panel Studies

Stimulus	Dose
Serum Free	
IL-4	50 ng/mL
LPS	1 µg/mL
NRG-1	100 ng/mL
LPS + IL-4	1 µg/mL LPS +50 ng/mL IL-4
LPS + NRG-1	1 µg/mL LPS + 100 ng/mL NRG-1

Cell Culture

SIM-A9 cells were grown according to methods outlined in Chapter 2 section

Methods – Cell Culture. For gene expression panel studies, cells were

always plated directly into media containing induction factors at

concentrations listed in Table 3-1 and samples collected following 24 hours

exposure.

RNA sample Isolation and Preparation

RNA samples were collected from cultured cells via direct lysis with buffer

RLT (Qiagen) in culture plates followed by homogenization through a 26-

gauge needle. Homogenates were stored immediately at -80°C until needed.

Total RNA was extracted from cell homogenates using on-column genomic

DNA digestion followed by isolation and purification of RNA using RNeasy

spin columns included in the Qiagen RNeasy plus mini-prep kit, as per the

manufacturer's instructions. Concentration of purified RNA was measured via

Qubit 2.0 fluorometer using the Qubit BR RNA assay kit while purity was

quantified using spectrophotometry (NanoDrop™ 2000, Thermo Scientific™) to determine 260/280 and 260/230 ratios, which assess protein and chemical contamination, respectively.

NanoString Methodology

NanoString is a semi-automated, medium throughput platform that can be used to look at gene and protein expression profiles and incorporates bioinformatics tools to analyze differential gene expression and gene ontology (GO)/ KEGG pathway analysis. The technology utilizes fluorescent barcodes hybridized to probes of known target molecules and single-molecule optical counting to provide count data on supplied samples. In this work, we used NanoString to obtain count-level transcription levels as this methodology offers some technical advantages over other counting techniques which also utilize RNA input, including RNA-seq and qPCR methods. NanoString does not require amplification of the RNA sample, which leads to more accurate read counts and the optical counting method provides exact, target-specific read counts, eliminating the need for technical replicates.

RNA was extracted from 6 biological replicates for each of the 6 induction protocols and at two timepoints (24-hour exposure and 6-hour exposure) as outlined in the manufacturer's instructions (NanoString MAN-10051-03). Samples meeting NanoString recommendations of 260/280 of ≥ 1.9 and 260/230 between 1.8 and 2.3 were assessed for quality using an Agilent

2100 bioanalyzer to determine RNA integrity number (RIN). The RIN was developed by Agilent Technologies using an algorithm generated by taking hundreds of samples and having specialists manually assign them all a value of 1 to 10 based on their integrity, with 10 being the highest. For all samples used, RIN > 8.0. Total RNA samples were diluted to concentration of 10ng/μl in RNase-free water immediately prior to hybridization to probes, as outlined in the code set hybridization setup manual (NanoString MAN-10056-02). We used the XT formulation mouse inflammation panel MmV2 which consists of 248 endogenous genes and 6 housekeeping genes for normalization. An amount of 50 ng of total RNA (5 μl) were used in hybridization reactions which were run for 18 hours at 65°C and left at 4°C no more than 4 hours. Hybridized samples were immediately loaded into cartridges and run on a NanoString SPRINT machine.

NanoString analysis: Normalization and Differential Expression

NanoString files of file type .RCC were downloaded at the completion of the run and prepared for analysis as outlined in the NanoString gene expression data analysis guidelines (NanoString MAN-C0011-04). Initial normalization of transcript counts and generation of expression ratios were performed using NanoString nCounter (nSolver 4.0, NanoString MAN-C0019-18) software. Additional analysis was done through the NanoString Advanced analysis 2.0 plugin (NanoString MAN-10030-03), which uses open-source and well-

characterized R programs to check QC, perform normalization, differential

expression analysis, pathway scoring and gene set enrichment analysis.

Normalization was performed by the advanced analysis algorithm, which

picks housekeeping genes from a designated set of potential housekeepers.

Housekeepers to utilize are chosen based on minimizing variation and are

chosen in a pair-wise fashion until a minimum variability is found.

 Differential gene expression was explored using two orthogonal analyses.

nSolver defines gene expression as the total signal – signal noise, and

models gene expression as a negative binomial distribution taking covariates

into account. Covariates consist of predictors and confounders. For these

experiments, treatment was the predictor and experiment was set as a

confounder to control for (eliminate) differences between independent

experiments.

Panel Analysis

Differential gene expression was evaluated using nSolver Advanced analysis

software which implements an algorithm modeling gene expression through

negative binomial regression and returns the p-value corresponding to the

likelihood that a population's gene expression is the same as the baseline

expression.

<u>Statistics</u>

The present study is exploration, so there is an advantage to including a

maximal portion of the data and minimal advantage to setting stringent limits

on p-value significance. As the number of comparisons made within the same set of data increases, so does the likelihood of false positives. To mitigate this issue we worked under the following criteria:

-Do not assume equal variance across all genes.

-Use non-pooled variance which includes only measurements of the expression of one gene.

- Limit comparisons to 6 pre-determined conditions (Table 3-2).

Table 3-2
List of Comparisons Between Conditions

SF vs LPS	LPS vs LPS + IL-4	IL-4+LPS vs NRG+LPS
SF vs IL-4	LPS vs NRG-1 + LPS	
SF vs NRG-1		

Decisions regarding significance were made based on the uncorrected p-values. However, for completeness and the benefit of those building on this work, we will report the uncorrected p-values as well as corrected p-values. P-value correction will be done by limiting the false discovery rate (FDR) utilizing the two-stage step-up procedure proposed by Benjamini, Krieger, and Yekutieli (Benjamini, Krieger et al. 2006), setting Q-value to 10. This limits the false positives to 10% of reported discoveries.

RESULTS AND DISCUSSION
Gene Expression Analysis

Raw count numbers were imported to nSolver and used to begin advanced analysis. Based upon the negative controls for all samples, the minimum count threshold was set at >20 counts and genes in which all samples fell below this threshold were removed from analysis.

Thresholding removed 63 genes from the inflammation panel leaving 185

genes (Table 3-3).

Table 3-3
Proportion of Genes Used in Analysis

# Genes	Inflammation Panel
Total	248
Above Cutoff	185
Removed	63

NRG-1 Exposure Induces Minimal Inflammatory Gene Expression in

Unstimulated SIM-A9 Microglia

To compare individual exposure conditions, fold changes are in reference to

unstimulated (serum free) control.

LPS showed changes in the majority of panel genes, and over 80% of

significant changes were upregulations (Table 3-4; Figure 3-1), indicating that

pro-inflammatory LPS treatment triggered gene expression of a large

proportion of inflammatory genes. IL-4 exposure altered roughly half the

number of genes as LPS, and effects were split more evenly between

upregulation and downregulation of genes (Table 3-4; Figure 3-1), indicating

that anti-inflammatory IL-4 stimulation induces an increase in some

inflammatory genes while preventing induction of others. NRG-1 exposure

resulted in the fewest significant gene expression changes of the three

individual stimulations tested, with 41 differences split about equally between

19 (46.3%) upregulated and 22 (53.6%) were downregulated genes. The

inflammatory gene response to NRG-1 was about a quarter the size of LPS

response and half the size of response to IL-4 in terms of number of genes involved.

Table 3-4
Gene Expression Changes Relative to Unstimulated Baseline Induced by Exposure to Individual Stimuli

	LPS	IL-4	NRG-1
Total	154	85	41
Upregulated (%)	128 (83.1%)	41 (48.2%)	19 (46.3%)
Downregulated (%)	26 (16.9%)	44 (51.8%)	22 (53.7%)

In addition, volcano plots (Figure 3-1) indicate that expression changes induced by NRG-1 were generally smaller in magnitude. The greatest NRG-1 induced gene expression upregulation was 1.69 fold more than unstimulated cells and the greatest NRG-1-induced downregulation was -5.81 fold. In contrast, LPS stimulation induced changes ranging from 7090-fold increase to -25.25 fold decrease and IL-4-induced expression changes ranged from 783 fold increase to -5.26 fold decrease, when compared to a baseline of unstimulated cell gene expression.
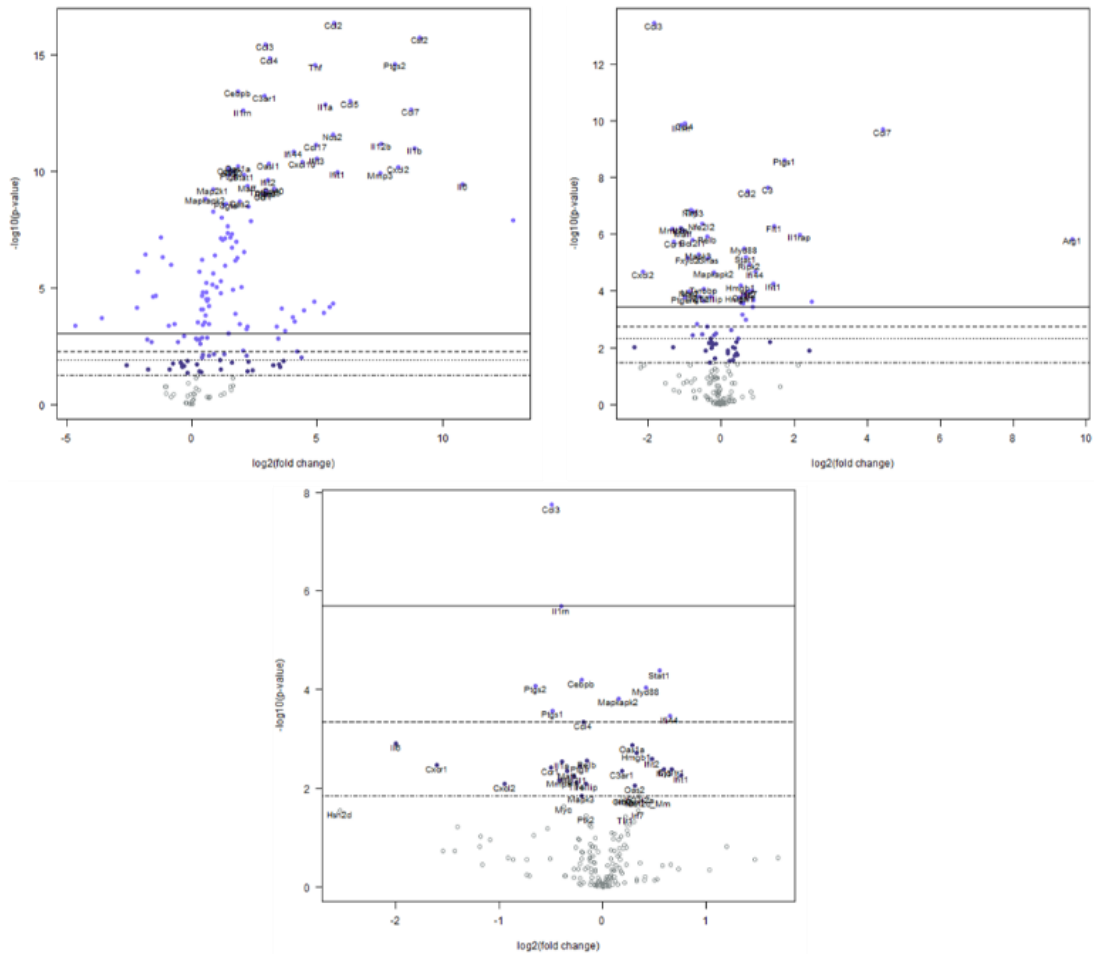
Figure 3-1 Volcano plots indicate that NRG-1 treatment alone in not a strong activator of inflammatory markers

Comparing volcano plots of individual molecule treatments shows clear differences in gene expression changes for this panel of inflammatory genes. Data plotted as $\log_2$fold change (or number of doublings) vs significance. Significance is calculated using raw p-value before BY correction for multiple comparisons. Lines indicate BY-corrected p-values. Top 50 significantly different genes in each treatment are labeled with gene symbol. Grey spots are not significantly different from baseline (p> 0.05); colored spot indicates significant difference (p<0.05). LPS (panel A) induced the widest range of fold changes including both strong upregulation and downregulation of genes within the dataset. Anti-inflammatory IL-4 (panel B) shows strong significant deviations from baseline, but skews to upregulation with few genes downregulated. NRG-1 (panel C) shows much narrower range for fold change with far fewer significant gene expression changes, indicating that NRG-1 alone does not significantly change inflammatory gene expression, while both traditional inflammatory treatments induce highly significant upregulation of inflammatory genes, indicating clear inflammatory activation.

Only ten of the 41 genes differentially regulated by NRG-1 varied from unstimulated baseline by ±50%, with Ifit1, Cysltr1, Ifi44, Ifit3, and Stat1 being upregulated and Ptgs2, Cxcl2, Cxcr1, IL-6, and Hsh2d downregulated (Table 3-5). Upregulated genes Ifi44, Ifit1 and Ifit3 are involved in immune response to viruses and literature has reported these molecules negatively regulate apoptosis. Upregulated Cysltr2 is a protein-coding genes for a cell surface receptor activated by leukotriene-type signaling lipids. It is directly involved in inflammatory responses to antigens and through traceable author statement evidence is associated with the GO term "negative regulation of inflammatory response to antigenic stimulus". Stat1 expression is induced by pro-inflammatory stimulus, and most strongly associated with interpheron-γ stimulation and signaling. STAT1 is a transcription factor and in turn regulates many of the other genes with expression altered by NRG-1, including Ifi44, Ifit1, Ifit3, Ptgs2, and IL-6.

Table 3-5
Differentially Expressed Genes Showing at Least ± 50% Change Following Individual NRG-1 Treatment

Official Gene Symbol	Gene Name	Fold Change	P-value
Ifit1	Interferon-induced protein with tetratricopeptide repeats 1	1.69	0.0055
Cysltr1	Cysteinyl leukotriene receptor 1	1.58	0.0041
Ifi44	Interferon-induced protein 44	1.57	0.00034
Ifit3	Interferon-induced protein with tetratricopeptide repeats 3	1.51	0.0041
Stat1	Signal transducer and activator of transcription 1	1.46	4.17E-05
Ptgs2	Prostaglandin-endoperoxide synthase 2	-1.57	8.42E-05
Cxcl2	Chemokine (C-X-C motif) ligand 2	-1.93	0.0082
Cxcr1	Chemokine (C-X-C motif) receptor 1	-3.04	0.0034
Il6	Interleukin 6	-4.00	0.0012
Hsh2d	Hematopoietic SH2 domain containing	-5.81	0.029

Downregulated molecules include established pro-inflammatory markers IL-6, Ptgs2 (COX-2) and Cxcl2. Notably, NRG-1 was the only condition that reduced levels of IL-6, Ptgs1, and Ptgs2 (COX-2) or increase Grb2. Together these expression changes indicate that NRG-1 stimulation may be antagonistic towards some pro-inflammatory gene expression.

NRG-1 Caused Fewer Overall Changes in Gene Expression as Compared to Anti-Inflammatory IL-4

To further characterize the nature of the influence of NRG-1 over immune phenotype, we compared the 41 observed gene expression changes that resulted from NRG-1 stimulation to gene expression changes induced by benchmark activators of inflammatory phenotypes, using LPS-induced changes as a model of pro-inflammatory changes and IL-4-induced changes from unstimulated baseline as a model of anti-inflammatory activation.

We found that when delivered individually to unstimulated cells, LPS caused the most changes to genes within this panel of inflammatory genes and IL-4 cause far fewer changes, but most interestingly, NRG-1 resulted in the fewest changes within this gene panel. Specifically, with 41 gene expression changes, NRG-1 had only half the number of gene changes as IL-4's 85 and roughly one-quarter (25%) of the changes caused by LPS (Table 3-4).

To characterize the influence of NRG-1 as more pro-inflammatory or more anti-inflammatory, we compared the NRG-1 induced changes to those of LPS and IL-4 respectively. For the 36 genes which showed significant deviation from baseline in both conditions, they were regulated in the same direction for all but 4 genes (Table 3-6). IL-4 upregulated Ptgs1, Ptgs2 and IL-6 while NRG-1 downregulated all three. Conversely, IL-4 downregulated Mapkapk2 while NRG-1 upregulated this gene, however the fold change was small (less than ±0.5 fold) in both cases.

In cases where IL-4 and NRG are regulated in the same direction relative to serum free controls, IL-4 shows a greater magnitude of change in almost all cases. The outliers are Cysltr1 and Oas1a in the upregulated direction and Cxcr1 and Hsh2d in the downregulated direction.

<u>The Majority of NRG-1-Induced Changes in Inflammatory Gene Expression in</u>

<u>Unstimulated SIM-A9 Microglia are Shared with IL-4</u>

Thirty-four of the forty-one significantly altered NRG-1 influenced genes were significantly regulated by all three individual stimuli, allowing direct assessment of alterations of these genes, as shown in Table 3-6. Interestingly, thirteen of these genes showed similar behavior for all three treatments. This included several of the genes most highly upregulated by NRG-1, and may indicate genes that are generally important for activation of inflammation and inflammatory activity, which may be necessary in both pro- and anti-inflammatory contexts.

However, for the overwhelming majority (17 genes out of 34), NRG-1 treatment resulted in alterations in gene expression in the same direction as anti-inflammatory IL-4 treatment and opposite direction from pro-inflammatory LPS. Only one gene showed the reciprocal behavior, with LPS and NRG-1 treatment going in the same regulatory direction and IL-4 causing the opposite. Finally, there were three genes for which NRG-1 behaved in the opposite manner as both IL-4 and LPS. Interestingly, these genes are all common markers of inflammatory activation. What is indicated by these results is that NRG-1 shared a greater proportion of its effects with IL-4 treatment than with LPS. In addition, NRG-1 caused few unique changes.

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Table 3-6
Regulation of Genes with Significant Expression Changes in All Individual Exposure Conditions

All (34)							
All Same direction (13)		NRG unique (3)		NRG like LPS (1)		NRG like IL-4 (17)	
↑	↓	↑	↓	↑	↓	↑	↓
12	1	3		1		4	13
Daxx	Cxcr1	Il6		Mapkapk2		Cysltr1	Bcl2l1
Ifi27l2a		Ptgs1				Hmgb1	Ccl3
Ifi44		Ptgs2				Hmgn1	Ccl4
Ifit1						Mef2c_Mm	Ccr1
Ifit2							Cxcl2
Ifit3							Hsh2d
Irf7							Il1a
Myd88							Il1rn
Oas1a							Maff
Oas2							Mapk3
Stat1							Myc
Tlr1							Relb
							Tollip

Key: ▮Regulated in same direction for all conditions; ▮Regulated same between LPS and NRG; ▮Regulated same between IL-4; ▮Unique Regulation by NRG-1

<u>Tlr4 Is Uniquely Significant and Downregulated by NRG-1 Stimulation Individually</u>

One gene that was significantly different in NRG-1 treatment condition alone, and not in either pro-inflammatory LPS treatment or anti-inflammatory IL-4 was Tlr4, an important regulator of pro-inflammatory signaling (Table 3-7). Tlr4 was upregulated by NRG-1, which may indicate that NRG-1 has a unique ability to directly alter inflammatory signaling through Tlr4. In addition, NRG-1 was the only treatment in which Tlr4 levels were significantly different with a slight reduction from baseline. Tlr4 is the receptor through which LPS mediates it signaling, so a reduction in Tlr4 expression has the potential to

make cells less sensitive to pro-inflammatory signaling through those receptors and would likely result in lower levels of inflammatory activation. Furthermore, LPS treatment showed a non-significant downregulation of Tlr4, and there have been additional literature reports that LPS downregulated its own receptor (Tlr4). NRG-1 may have the ability to regulate this important inflammatory receptor.

Table 3-7
Gene Expression Patterns for Genes Significantly affected by NRG-1 treatment

NRG (1)		NRG And LPS (4)				NRG And IL-4 (2)			
		Same (1)		Opposite (3)		Same (2)		Opposite	
↑	↓	↑	↓	↑	↓	↑	↓	↑	↓
		1		1	2		2		
Tlr4		C3ar1		Grb2	Cebpb Ptgir		Mmp9 Ptk2		

Key: When sig. for LPS and NRG-1 only: ▇ Regulated same between LPS and NRG-1; ▇ Regulated oppositely between LPS and NRG-1
When sig. for LPS and NRG-1 only: ▇ Regulated same between IL-4; ▇ Regulated oppositely between IL-4 and NRG-1
When significant for NRG-1 ONLY: ▇ Upregulated ▇ Downregulated by NRG-1

Six additional genes were regulated in the opposite directions (relative to control). Of these, Grb2 was the only one that was uniquely upregulated. Grb2 in involved in ErbB signaling as well as inflammatory activation. The remaining five genes uniquely regulated by NRG-1 and not the other two treatments were all downregulated in NRG-1 treatment. These genes included Cebpb, which is unaffected by IL-4 but largely increased by LPS. Cebpb binds particular DNA regulatory elements that can turn on multiple cytokines including IL-6, IL-4, and TNF-alpha and is necessary for

macrophage differentiation. These are largely pro-inflammatory roles, therefore NRG-1 downregulating expression may indicate a shift towards anti-inflammatory activation. Ptgir, Ptgs1 and Ptgs2 were all downregulated, and are all members of the prostanoid signaling family, indicating that NRG-1 treatment may be antagonistic to prostanoid signaling. Finally, the most prominently oppositely regulated gene was IL-6. IL-6 is generally used as an indicator of inflammation, but the increase under IL-4 stimulation indicated that it is also present in other types of microglial activation. NRG-1 treatment strongly downregulates IL-6 production, which could be interpreted as reducing microglial activation generally and possibly proinflammatory activation in specific. Genes which showed significant changes only in NRG-1 but neither pro- nor anti-inflammatory activations consisted of effects that could be interpreted as driving cells towards a more pro-inflammatory state or a more anti-inflammatory state, but there was not a clear tipping point in either direction. Therefore, NRG-1 may, in fact, be acting in a unique way that balances these two extremes. A summary of all unique NRG-1 genes and their regulation is shown in Table 3-8

Table of Gene Expression Changes Observed for NRG-1 Treatment Only

Table 3-8
Table of Gene Expression Changes Observed for NRG-1 Treatment Only

	LPS	IL.4	NRG
Tlr4			-
Cebpb	++		-
Grb2	-		+
Il6	+++	+++	---
Ptgir	++		-
Ptgs1	+	++	-
Ptgs2	+++	+	-

+ log2 fold change between 0 and 1   - log2 fold change between 0 and -1
++ log2 fold change between 1 and 2 -- log2 fold change between -1 and -2
+++ log2 fold change greater than 2   --- log2 fold change less than -2

NRG-1 Regulation of Inflammatory Gene Expression Changes Following Combined Exposure of SIM-A9 Cells with NRG-1 and LPS

The previous sections investigated treatment of unstimulated SIM-A9 microglia with individual factors of either pro-inflammatory LPS, anti-inflammatory IL-4, or NRG-1. In this section we will look at how NRG-1 treatment alters gene expression in the presence of an inflammatory stimulus. To understand the effect of NRG-1 within an inflammatory context, cells were simultaneously exposed to a combination of pro-inflammatory induction molecule LPS and a secondary stimulus of either anti-inflammatory IL-4 or NRG-1 at the same concentrations used in previous individual factor treatment experiments. In analysis of individual treatments, unstimulated (serum free) cells offered a useful baseline. However, unstimulated cells were not an adequate baseline to investigate combined treatments.

As seen in Table 3-4, LPS induces far more gene expression changes within this panel than IL-4 or NRG-1. The number of gene expression changes from

unstimulated cells induced by combination treatments are similar in

magnitude to those for LPS (**Error! Reference source not found.**),

indicating that LPS is likely driving the majority of gene expression changes

in combined treatments, however the baseline of unstimulated cells obscures

the contributions of each factor individually. Comparing only to unstimulated

cells ignores possible unique effects of combined treatment because it does

not indicate if combined treatment results in a third level of gene expression

that is significantly different form both unstimulated cells and LPS individual

treatment. To separate the changes due to LPS from the changes due to the

other factor in the combined treatment (IL-4 or NRG-1), we used the LPS

individual treatment dataset as a baseline (**Error! Reference source not

found.**), thus looking at how the addition of the second treatment alters the

effects of LPS individual treatment.

Table 3-9
Stimulus-Induced Inflammatory Gene Expression Changes

Baseline	LPS	IL-4+LPS	NRG-1+LPS
Unstimulated (Serum Free)	154	136	142
LPS	--	94	39

Volcano plots offer a visual representation of how the change in baseline

elucidates distinctly different effects of NRG-1 and IL-4 (Figure 3-2). The

upper plots using unstimulated cells as a baseline look extremely similar for

LPS+IL-4 and LPS+NRG-1, while lower plots using LPS as a baseline

highlight just the effects of IL-4 or NRG-1 within the combination treatment

and show a clear visual distinction in regulation behaviors. Specifically, the

upper panels show a range of fold changes (the x-axis) spanning from -5 to +15, or heavily weighted towards upregulations, for both LPS+IL-4 and LPS+NRG-1. In contrast, the lower panels show that LPS+IL-4 has a fold change range from -2 to+6, with the majority of changes clustering in the negative fold change range and a few high magnitude upregulations, such as Arg1. The lower panels also showcase that LPS+NRG-1 results in a very distinctly different behavior, with an almost symmetrical spread of upregulations and downregulations.
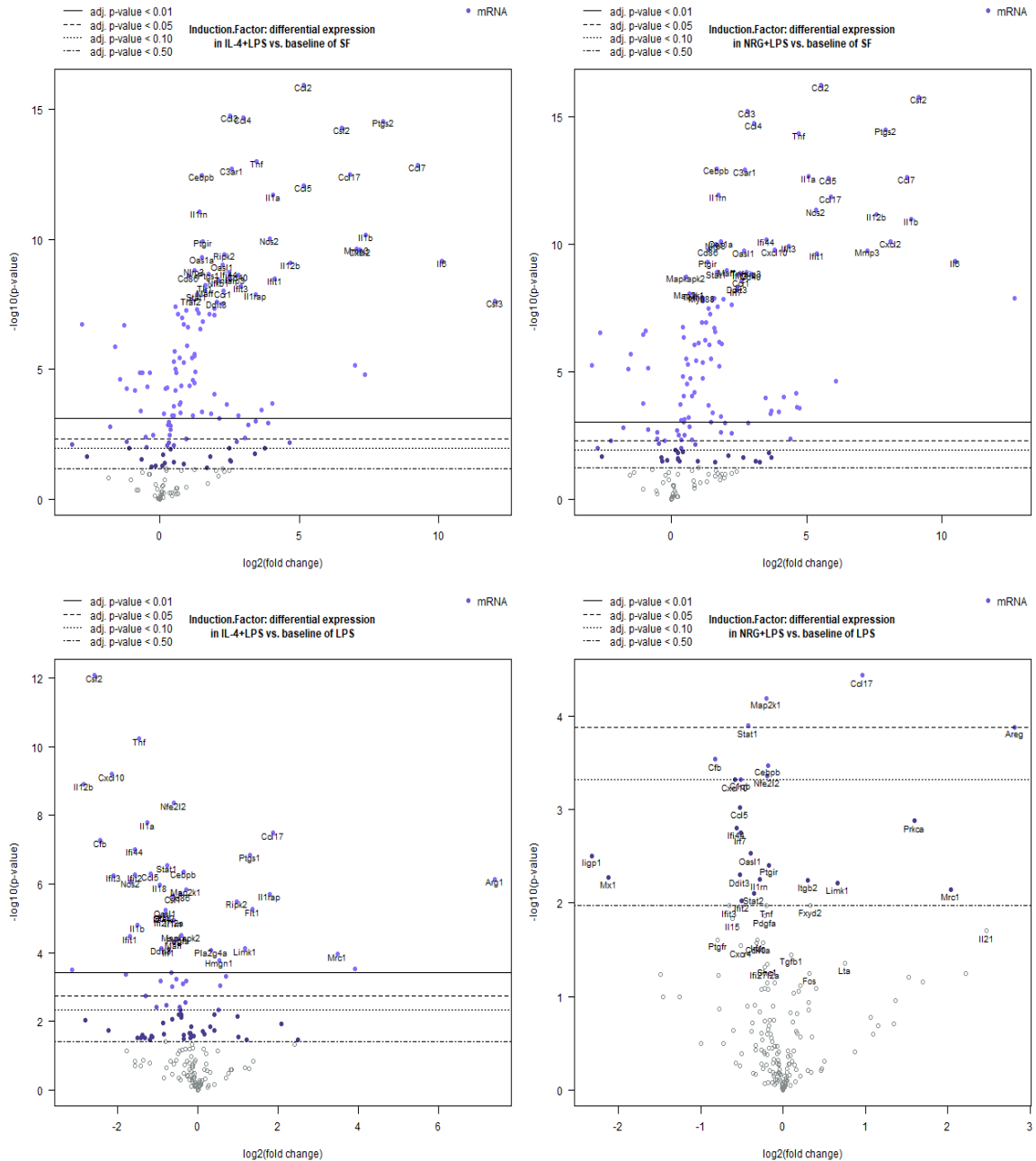
Figure 3-2 Volcano plots comparing combined treatments

The upper panels show gene expression compared to a baseline of serum free unstimulated cells. The lower panels show differential expression as compared to LPS treatment. Using LPS as a baseline showcases that IL-4 alters the expression of more genes than NRG-1, but the magnitude of up or down regulation is comparable.

There was a total of 39 expression changes between individual LPS and

combined LPS + NRG-1, consisting of 10 genes which were upregulated

relative to LPS and 29 genes that were downregulated (Table 3-10). Many of these changes were relatively small, so when including only $\log_2$ fold changes ± 1 $\log_2$ fold, only six gene expression levels changed, four upregulated and two downregulated.

Table 3-10
Changes in LPS-induced Gene Expression with the Addition of Secondary Factor

Differentially Regulated Genes	IL-4 + LPS	NRG + LPS
Total	94	39
Upregulated (%)	25 (27%)	10 (26%)
Downregulated (%)	69 (73%)	29 (74%)

Using LPS as a baseline suggested a different analysis of gene expression change to that used for evaluation of individual treatments. To evaluate individual treatments, we only looked at upregulation or downregulation relative to unstimulated (serum free) baseline. For combination treatments, we defined change in reference to LPS-induced gene expression changes from an unstimulated baseline, rather than the direction of the expression change. The new definition of change has three possible states:

1. **Reversed gene expression change**: When combination treatment results in gene expression change that is in the opposite direction to LPS-induced expression change from baseline. In this case, the gene expression level of the combination treatment will be closer to the unstimulated condition than LPS gene expression level is to the unstimulated condition. Reversed gene expression can occur via two pathways.

a. *Up-Down*: LPS individually increases expression relative to unstimulated control level, then combination treatment decreases expression relative to LPS

b. *Down-up*: LPS individually decreases expression level relative to unstimulated control, then combination treatment increases expression level relative to LPS

In a *perfect reversal*, the combination treatment will be significantly different from individual LPS gene expression level but not significantly different from unstimulated condition gene expression level.

2. **Enhanced gene expression change**: When combination treatment results in gene expression change that is in the same direction as the LPS-induced change. Enhanced gene expression can occur in two ways

a. *Up-Up*: LPS individually increases expression relative to unstimulated control level, then combination treatment further increases expression level relative to LPS

b. *Down-Down*: LPS individually decreases expression relative to unstimulated control level, then combination stimulus further decreases expression relative to LPS

For genes with enhanced gene expression change, the change relative to unstimulated cells is in the same direction for LPS and combination

treatment, but the combination treatment change is greater in magnitude.

3. **Unique gene expression change**: When individual LPS treatment did not result in significant gene expression change relative to serum free baseline but combination treatment gene expression level is significantly different from LPS individual treatment expression level. Unique gene expression can be either upregulated relative to LPS or downregulated relative to LPS. In addition, it is possible for the combination expression level to be significantly different from unstimulated cell control level or not significantly different relative to control. Figure 3-3 shows a flow diagram outlining how these gene set categories are determined.
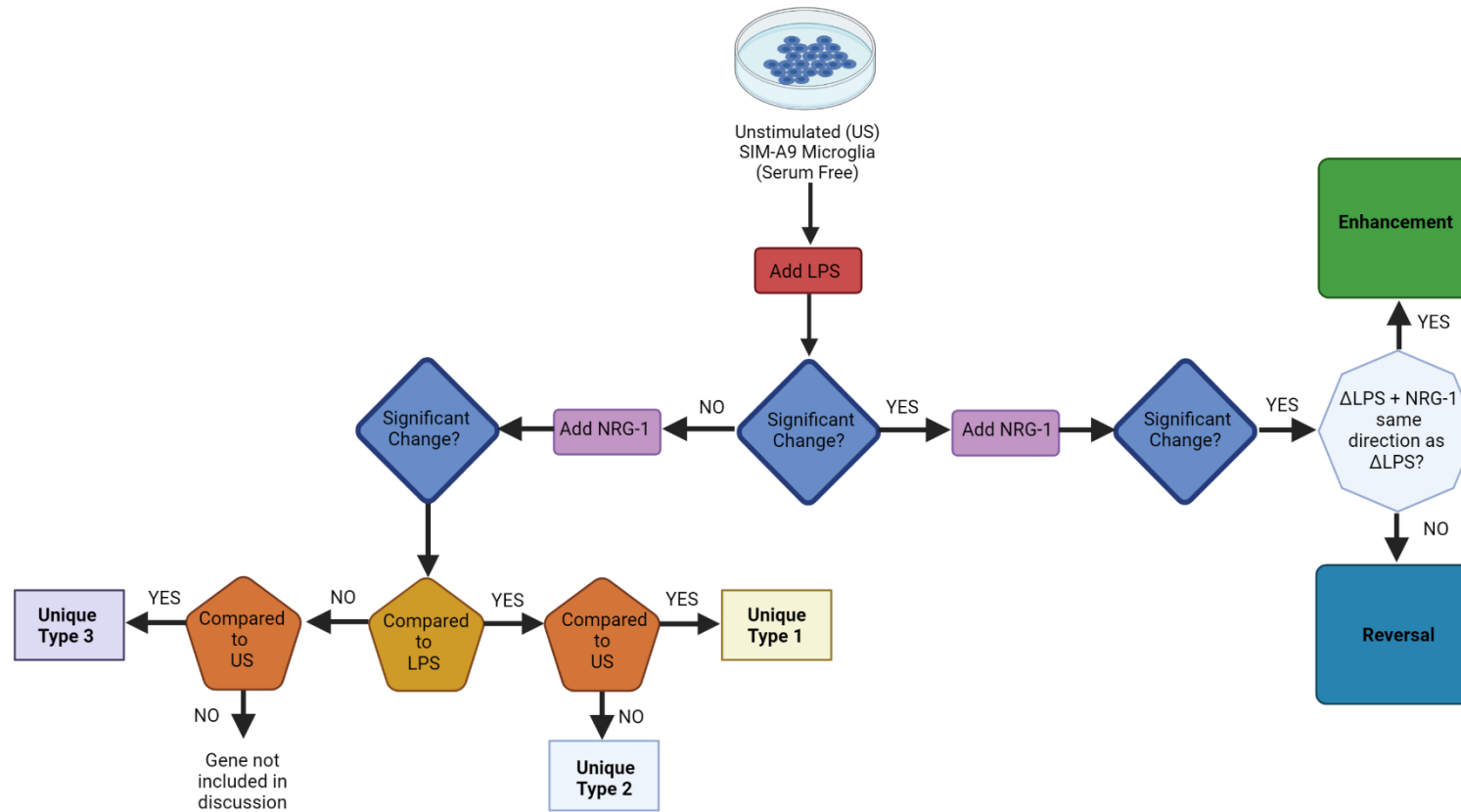
Figure 3-3 Flow chart outlining gene set categorization
Starting with unstimulated cells, flow chart outlines classification of gene expression change based upon significant expression change resulting from addition of stimuli LPS and NRG-1.

Lists of reversed, enhanced, and unique gene expression changes give insight into the changes resulting from combination treatment. Comparing these lists for LPS + IL-4 treatment and LPS+NRG-1 treatment shows which gene expression changes are unique to LPS+NRG-1. Identifying unique NRG-1 induced changes may elucidate regulation mechanisms unique to NRG-1, while the shared gene expression changes between LPS+NRG-1 and LPS + IL-4 datasets point to shared mechanisms with canonical anti-inflammatory processes.

Tracking the regulatory mechanisms above for this dataset, we see that NRG-1 has minimal influence on inflammatory gene expression changes (Table 3-11,
Table 3-12). NRG-1 results in 39 significant alterations from individual LPS exposure, while the exposure to anti-inflammatory IL-4 induces over twice that number of inflammatory gene expression changes with 84 total differences (Table 3-11). Additionally, the response that NRG-1 does have (39 genes), almost completely overlaps with the IL-4 influence (84 genes), with only seven of the inflammatory genes responding to NRG-1 in a unique way (Table 3-11Table 3-11 and
Table 3-12).

Table 3-11
Total Inflammatory Panel Gene Expression Changes induced by Combination Treatments

		Complete Response					
		NRG-1		IL-4		Shared	
Reverse	Up	31	4	62	7	29	3
	Down		27		55		26
Enhance	Up	4	3	14	12	2	1
	Down		1		2		1
Unique	Up	4	3	8	6	1	1
	Down		1		2		0
Total		39		84		32	

Table 3-12
Stimulus-Specific and Shared Inflammatory Panel Gene Expression
Changes induced by Combination Treatments

		Unique Response					
		NRG-1		IL-4		Shared	
Reverse	Up	2	1	33	4	29	3
	Down		1		29		26
Enhance	Up	2	2	12	11	2	1
	Down		0		1		1
Unique	Up	3	2	7	5	1	1
	Down		1		2		0
Total		7		52		32	

<u>Reversal of LPS-induced Gene Changes by NRG-1 and IL-4</u>

Thirty-one genes were Reversed, meaning that they changed in a way that was towards baseline (Table 3-13). The majority of these were downregulated in the presence of NRG-1 after, but four were upregulated. Two of these genes were changed by NRG-1 alone and not IL-4. Itgb2 is a plasma membrane protein that serves as an integrin and is upregulated by IL-13 exposure, indicating a more anti-inflammatory response.

Table 3-13
Inflammatory Panel Genes Reversed By Combination Treatment

Gene Symbol	Gene Name	Fold Change	
		NRG-1+ LPS	IL-4+ LPS
Itgb2	Integrin beta 2	1.23	-1.08
Ptgir	Prostaglandin I receptor (IP)	-1.13	1.02
Limk1	LIM-domain containing, protein kinase	1.58	2.25
Mrc1	Mannose receptor, C type 1	4.12	11.10
Prkca	**Protein kinase C, alpha**	3.04	2.00
Ccl5	Chemokine (C-C motif) ligand 5	-1.45	-2.27
Cd40	CD40 antigen	-1.25	-1.37
Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	-1.14	-1.29
Cfb	Complement factor B	-1.78	-5.38
Cxcl10	Chemokine (C-X-C motif) ligand 10	-1.50	-4.48
Ddit3	DNA-damage inducible transcript 3	-1.44	-1.88
Ifi27l2a	Interferon, alpha-inducible protein 27 like 2A	-1.17	-1.66
Ifi44	Interferon-induced protein 44	-1.48	-2.98
Ifit2	Interferon-induced protein with tetratricopeptide repeats 2	-1.42	-2.97
Ifit3	Interferon-induced protein with tetratricopeptide repeats 3	-1.58	-4.29
Iigp1	Interferon inducible GTPase 1	-5.03	-8.85
Il15	Interleukin 15	-1.53	-1.72
Il1a	Interleukin 1 alpha	-1.18	-2.40
Il1rn	Interleukin 1 receptor antagonist	-1.22	-1.53
Irf1	Interferon regulatory factor 1	-1.25	-1.65
Irf7	Interferon regulatory factor 7	-1.43	-1.94
Map2k1	Mitogen-activated protein kinase kinase 1	-1.15	-1.23
Mx1	**Myxovirus (influenza virus) resistance 1**	-4.33	-2.60
Nfe2l2	Nuclear factor, erythroid derived 2, like 2	-1.14	-1.51
Oasl1	2'-5' oligoadenylate synthetase-like 1	-1.32	-1.76
Pdgfa	Platelet derived growth factor, alpha	-1.17	-1.41
Ptgfr	Prostaglandin F receptor	-1.74	-2.46
Shc1	Src homology 2 domain-containing transforming protein C1	-1.14	-1.25
Stat1	Signal transducer and activator of transcription 1	-1.34	-1.71
Stat2	Signal transducer and activator of transcription 2	-1.28	-1.79
Tnf	Tumor necrosis factor	-1.15	-2.74

Genes above the double line are enhanced by NRG-1 treatment only and below the double line are enhanced by both IL-4 and NRG-1 treatments.
Green indicates upregulation compared to a baseline of LPS
Red indicates downregulation compared to a baseline of LPS
Grey indicates p-value for difference is not significant
Bold denotes genes where NRG-1 treatment resulted in a greater magnitude gene expression change than IL-4

Enhancement of LPS-Induced Gene Expression

Rather than rescuing, in four cases genes were actually altered further in the direction that LPS had regulated these genes. For the purposes of our analysis, this type of regulation was referred to as enhancement.

The gene set in our panel that is enhanced by NRG-1 treatment consists of four (4) genes, with half of that enhancement response (two genes) being unique to NRG-1 and half is shared with anti-inflammatory IL-4 (Table 3-14). Both uniquely enhanced NRG-1 genes were upregulated in LPS and further upregulated with NRG-1 treatment, while shared enhanced genes include Ccl17 which was further upregulated and Cxcr4 which was further downregulated.

Table 3-14
Addition of NRG-1 Treatment Further Enhanced LPS-Induced Changes in Inflammatory Gene Expression

Gene Symbol	Gene Name	Fold Change	
		NRG-1+ LPS	IL-4+ LPS
Lta	Lymphotoxin A	1.69	-2.19
Tgfβ1	Transforming growth factor, beta 1	1.07	-1.07
Ccl17	Chemokine (C-C motif) ligand 17	1.94	3.68
Cxcr4	Chemokine (C-X-C motif) receptor 4	-1.43	-1.57

Genes above the double line are enhanced by NRG-1 treatment only and below the double line are enhanced by both IL-4 and NRG-1 treatments.
Green indicates upregulation compared to a baseline of LPS
Red indicates downregulation compared to a baseline of LPS
Grey indicates p-value for difference is not significant

Lta and Tgfβ1 were enhanced by the addition of NRG-1 when compared to LPS alone, and this enhancement behavior was exclusive to NRG-1 because the addition of IL-4 to LPS stimulation resulted in significant reversal of LPS-induced changes rather than enhancement. In addition, both of these

exclusive NRG-1 enhancements were NRG-1-induced upregulations from baseline of LPS and unstimulated baseline. Correspondingly, under IL-4 stimulation these two gene changes were reversals and downregulations from LPS, but were not perfect reversals because they were still upregulated with respect to unstimulated baseline. Lta is a pro-inflammatory cytokine and member of the tumor necrosis factor family, which is secreted and binds TNFRs, where it plays a role in activating inflammation and is an important regulator for cell survival and initiating apoptotic signaling. Lta is associated with the apoptotic pathway and is involved in TNFR2 initiated non-canonical NF-κB pathway. Tgfβ1is highly involved in microglial function and neurodegeneration. Tgfβ1 signaling is necessary for microglia to respond and adapt to changes in the surrounding environment.

Unique Gene Expression Changes to NRG-1 Treatment within an Inflammatory Context

In some cases, genes that were not significantly changed from baseline through LPS alone did show significant differences from baseline when LPS and NRG-1 treatments were delivered concurrently. These genes represent a unique subset of genes that are influenced by NRG-1, but only when microglia are within microglia are in a state of inflammatory activation. The existence of this subset genes that change only for combinatorial treatment but not either individual treatment (NRG-1 only or LPS only) is a strong indicator that NRG-1 is an inflammomodulatory molecule. Notably there is no

overlap in this set of genes uniquely changed by combined NRG-1 + LPS treatment and the set of genes changed by NRG-1 alone, which may point to NRG-1 having different effects or working though different mechanisms depending upon the inflammatory state activation of the microglial cell. There were four genes that were not affected by LPS alone (no significant difference in expression with reference to baseline of serum free cells) but were changed when NRG-1 treatment was added to LPS stimulation (Table 3-15).

Table 3-15
Significant Gene Expression Changes Following Combined NRG-1+ LPS Treatment That Were Not Induced Through LPS Treatment

Gene Symbol	Gene Name	Fold Change	
		NRG-1+ LPS	IL-4+ LPS
Fxyd2	FXYD domain-containing ion transport regulator 2	1.26	1.15
Il21	Interleukin 21	5.56	-1.8
C1qb	Complement component 1, q subcomponent, beta polypeptide	-1.43	-1.16
Areg	**Amphiregulin**	6.99	2.32

Genes above the double line are enhanced by NRG-1 treatment only and below the double line are enhanced by both IL-4 and NRG-1 treatments
Green indicates upregulation compared to a baseline of LPS
Red indicates downregulation compared to a baseline of LPS
Grey indicates p-value for difference is not significant
Bold denotes genes where NRG-1 treatment resulted in a greater magnitude gene expression change than IL-4

IL-21 was most strongly induced by NRG-1 and its direction of regulation was opposite. IL-21 is generally a pro-inflammatory cytokine.

Fxyd2 has been reported as upregulated in IL-10 stimulated macrophages in vitro, which may indicate a role in inflammatory resolution or deactivation.

Cq1b is identified as a marker for identifying brain-derived microglia.

Areg is an EGFR ligand and expression in microglia is associated with increased cellular invasion. Most studies looking at Areg expression in microglia come out of work on tumor associated macrophages (TAMs). Areg is involved in wound healing and tissue repair, and intraventricular administration of Areg following stroke reduces neurotoxic gene expression astrogliosis and neuronal apoptosis and may be indicative of a brain-tissue-specific marker in immune cells (Ito, Komai et al. 2019). Areg may be involved in activation STAT3 and subsequent upregulation of IL-6 (Ito, Komai et al. 2019).

Magnitude of NRG-1-induced Changes to LPS Activation in Combined Stimulation with NRG-1 and LPS

The largest increases in the presence of NRG-1 were Areg (+6.99 fold), Il21 (+5.56 fold), and Mrc1 (+2.04 fold). Largest decreases include Iigp1 (-2.33) and Mx1 (-2.12). Iigp1 is a part of the A1 gene signature for neurotoxic reactive astrocytes.

Prkca may be anti-apoptotic through phosphorylating BCL2. In immune response to LPS, may negatively regulate NF-kB-induced genes, through IL1A-dependent induction of NF-kappa-B inhibitor alpha (NFKBIA/IKBA).
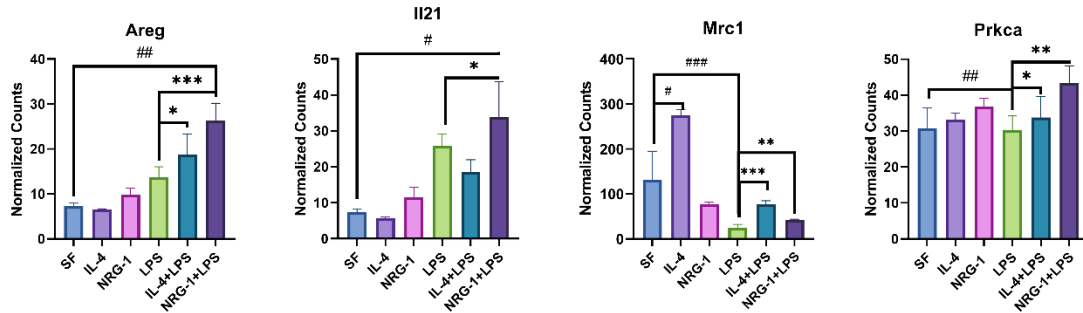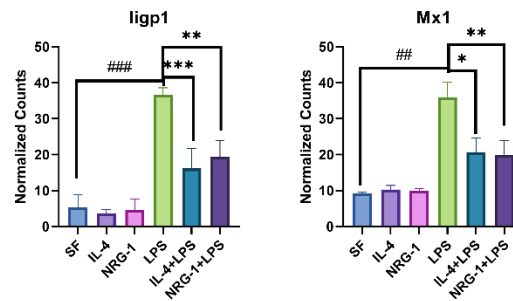
Figure 3-4: Gene counts for genes in which NRG-1 had the greatest effect when delivered concurrently with LPS.
P-values: Stars (*) compared to LPS; * >0.05, ** > 0.01, *** > 0.001. Hash(#) compared to SF control; # >0.05, ## > 0.01, ### > 0.001

The majority of these high difference genes have relatively low expression

with 50 or fewer counts. This is true for all of these genes except Mrc1. Three

of these genes, IL-21, Iigp1, and Mx1, were increased significantly by LPS

and in the latter 2 downregulated genes, NRG-1 was able to result in an

expression change towards baseline (reversal of LPS-induced change)

Amphiregulin (Areg) is related to epidermal growth factors and is associated

with some immune pathologies. IL-21 is a pro-inflammatory cytokine. Mrc1 is

also known as CD206, coding for mannose receptor type C. This is a marker

of the anti-inflammatory phenotype. Mx1 is involved in antiviral activity. Iigp1

is an interferon inducible GTPase with low activity and a higher affinity for

GDP and GTP that is required in resistance to parasite T. Gondii.

Alone, NRG-1 does not strongly stimulate any factors present in this panel.

Notably, it does not stimulate Arg1 which was strongly upregulated by IL-4,

both in the presence and absence of LPS

In the presence of LPS, NRG-1 reduces pro-inflammatory markers IL-6,

Nos2, Cxcl10, and Stat1 but does not affect IL-1b, IL12b, IL-2a, or Cd86. In

addition, the M2 marker Mrc1 is increased, as in IL-4. Cox2 expression is

significantly reduced in the presence of NRG-1 while this is only a trend in

the IL-4 condition. Areg and Ptgir are increased, which was unique to NRG-1.

The only significant effect that NRG-1 treatment had on LPS-induced effects

in this cells line were to the genes Ptgs2 and Ptgir. Ptgs2 (COX-2) codes for

prostaglandin-endoperoxide synthase 2, an enzyme that synthesizes

prostaglandin H2 from arachidonic acid. This is a common proinflammatory

marker and competes for the same substrate as anti-inflammatory marker

Arg1. Ptgir codes for prostenoid receptor prostaglandin I2 (prostacyclin)

receptor (IP). This is a G-protein coupled type receptor.

NRG-1 Induced Changes of Traditional Markers of Polarization

The previous section outlined how NRG-1 altered gene expression in

comparison to pro-inflammatory LPS and anti-inflammatory IL-4, establishing

that for this panel of inflammatory markers NRG-1 treatment alone minimally

activates inflammatory markers that the main effects of NRG-1 , but how

does this fit onto the context of traditional markers of inflammation and

polarization?

Table 3-16
Summary of Traditional Markers of M1 and M2 Polarization States

	M1	M2		
		M2a	M2b	M2c
Stimulus	LPS, IFNγ, LTA, GM-CSF (Csf2), dsDNA, ssRNA, unmethylated CpG islands	IL-4, IL-13	TLR and IgG	IL-10
Released Factors	TNF, IL-1β, IL-6, IL-12, IL-23, CCL2 (MCP1), CXCL10 (IP10)	IL-10, Polyamines	TNF, IL-1β, IL-6, IL-10	Matrix proteins, TGFβ, IL-10
Cell Surface Markers	CD86, CD40,CD16/32, Ccr1 MHCII	Mrc1 (CD206), SRs(CD163)	CD86, MHCII	SLAM, Mrc1(CD206)
Intracellular Markers	Nos2 (iNOS), Ptgs2 (COX-2)	Arg1*, YM1* (Chi3l3), Fizz1* (Retnla)		
Nucleus	STAT1/5	STAT6		STAT3

* Marker only for mice(Raes, Van den Bergh et al. 2005)
Abreviations: SRs – scavenger receptors

To put our results into context with the existing literature based on

biomarkers of inflammatory states, we need to evaluate our experiments

based upon typical M1 and M2 markers (Table 3-16), many of which were

present in the inflammation NanoString panel. We expect that we should see

an increase in M1 markers under the LPS condition and a reversal towards

baseline when anti-inflammatory IL-4 is delivered in concert with pro-

inflammatory stimulus LPS, effectively cancelling each other out.

Secreted Factors

Cell Surface Markers

Intracellular Markers

IL-4 Significantly Downregulates LPS-induced Upregulation, NRG1 Does Not Alter LPS-Induced Upregulation

Il1b

Il12b

Il18

Ccl3

Cd86

Nos2
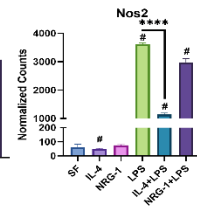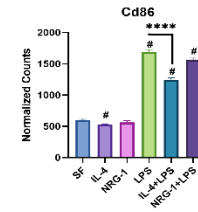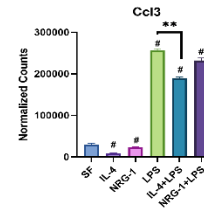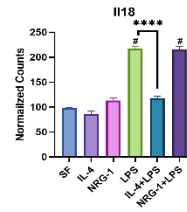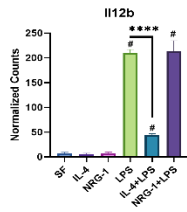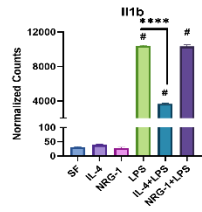
IL-4 and NRG-1 Significantly Downregulate LPS-induced Upregulation

Il15

Cxcl10

Tnf

Ccl5

Cd40
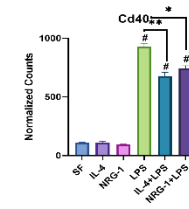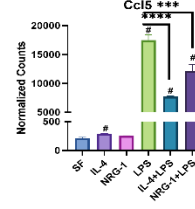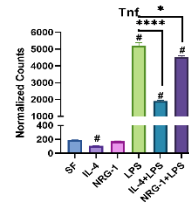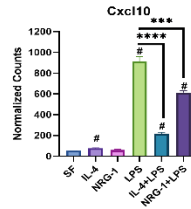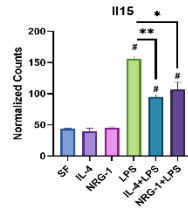
Nonsignificant Trend: IL-4 and NRG1 Reverse LPS-Induced Upregulation, but NRG1 Reduction Is Lesser In Magnitude

Il6

Il23a

Ccl2

Mmp3

Ccr1

Ptgs2
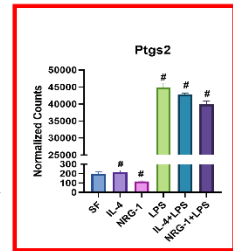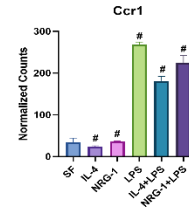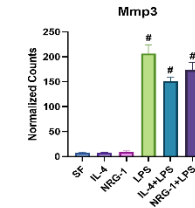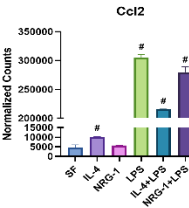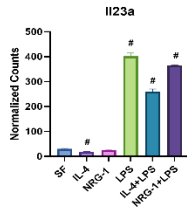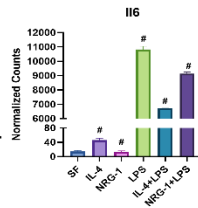
Figure 3-5 The effects of 100ng/mL NRG-1 on pro-inflammatory (Canonical M1) gene expression

Bar charts show normalized mRNA count number of M1 markers over six chemical induction conditions. Markers are grouped vertically by location within a cell or extracellular space and horizontally by expression pattern. Three major patterns of expression emerge. In the first row, IL-4 significantly downregulates LPS-induced upregulation of M1 marker while NRG-1 has no effect (expression for NRG-1+ LPS = expression for LPS). The second row shows a pattern where both IL-4 and NRG-1 significantly reduce LPS-induced upregulation of pro-inflammatory markers. For all cases, NRG-1 reduction is lesser in magnitude than that achieved with IL-4. The third row shows a pattern similar to the row above, however the differences in expression levels are trends that do not reach statistical significance. The three expression patterns are seen in markers spanning several different locations within the cell, indicating that these expression patterns influence all aspects of microglial inflammatory state, from effector and signaling functions (through secreted factors), to sensing abilities (through alterations in cell surface markers), and intracellular metabolic or processing (seen in alterations in intracellular markers). Bar graphs also demonstrate that NRG-1 individually (pink bars, third from the left) induced minimal expression change from SF for all markers shown, while IL-4 (light purple bars, second from left) exposure resulted in slight but significantly alternations in either the downregulated (Ccl3, TNF, IL23a, Ccr1) or upregulated (Cxcl10, Ccl5, IL8, Ccl2) direction. Ptgs2 does not fit into any of the expression patterns otherwise noted. Ptgs2 is a well-documented pro-inflammatory marker, but here NRG-1 downregulates Ptgs2 to a greater degree than IL-4 and under LPS-activated and non-activated conditions (NRG-1 + LPS and NRG-1). P-value: # baseline SF, *baseline LPS * = <0.05, **= <0.01, ***= <0.001, ****=<.0001, ns = not significant.

In this dataset we generally see the expected result, with LPS increasing M1 markers significantly while the addition of IL-4 reduces the factors down to baseline. Interestingly, in a few cases we see that in the absence of LPS, IL-4 causes a modest but significant increase in the M1 marker, while when IL-4 is delivered to activated LPS-induced cells, the effect is reversed. This pattern is evident in IL-6 and Ccl2, Ccl5, Cxcl10 and Ptgs2.

Comparing the effect of IL-4 to the effect of NRG-1, we see that there are similarities as well as distinct differences. While IL-4 reduces all M1 markers on this Nanostring panel, the effect of NRG-1 is more variable. For the majority of M1 markers, the addition of NRG-1 reduces LPS-induced upregulation of pro-inflammatory markers back towards baseline, but generally to a lesser degree than achieved by addition of IL-4. However, for a subset of M1 markers, NRG-1 has no effect on LPS-induced upregulation, resulting in no difference in expression level for LPS and NRG-1+LPS conditions. The ability for NRG-1 to selectively reduce expression of some inflammatory factors while maintaining expression of others could be an important feature of NRG-1 treatment. Mounting evidence shows that immune response is a double-edged sword: while it may cause short-term tissue damage, it is also necessary for long-term healing. Many therapies targeting the immune response have sought to shut it down or reduce it across the board. However, these strategies often fail. NRG-1 may be able to

efficiently target a subset of factors in the immune response, allowing more targeted immune modulation.

Expression of the following genes is not affected by NRG-1 treatment: IL-1β, IL-12b, IL-18, Ccl3, CD86, Nos2 (iNOS), Ccl7 and Csf2(GM-CSF). IL-1β and IL-12b are markers of pro-inflammatory states and Csf2 is thought to induce a pro-inflammatory state.

On the other hand, NRG-1 treatment did result in statistically significant downregulation of traditional pro-inflammatory markers IL-15, Ccl5, Cxcl10, CD40, and Tnf. Downregulation by NRG-1 was lower in magnitude than the level achieved by IL-4 in all of these cases. However, for important M1 marker Ptgs2 (Cox-2), NRG-1 lowered level further towards baseline than IL-4. Therefore, NRG-1 can be seen in these instances to be exhibiting an anti-inflammatory influence.

This panel also contained canonical M2 markers which we can use in order to see if NRG-1 induces expression of factors traditionally understood to be anti-inflammatory. Within this context, induction with IL-4 served as both a positive control and a benchmark "anti-inflammatory" stimulus to which NRG-1 was compared.

Several patterns of expression were seen within the M2 factors found in this panel, as can be seen in Figure 3-6. For secreted factors and cytokines, two expression patterns that we saw were that IL-4 induced expression in the

absence and presence of LPS- activation or IL-4 induced expression only

when also in the presence of LPS activation.
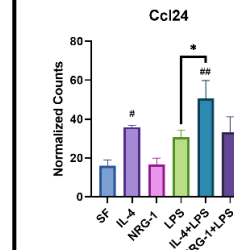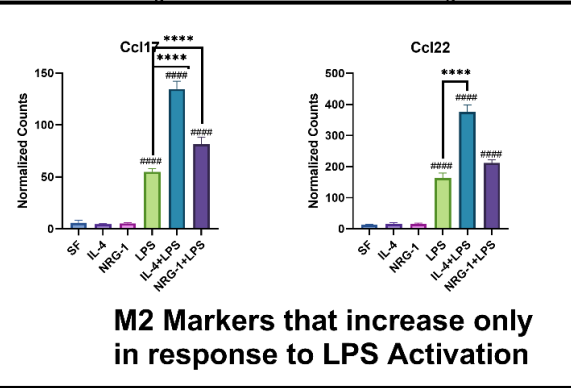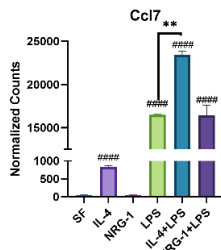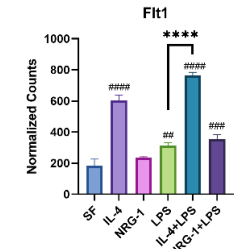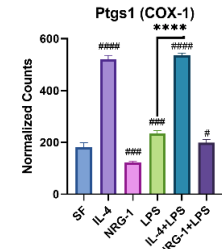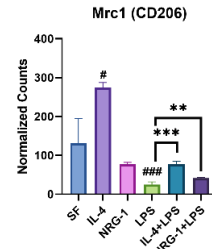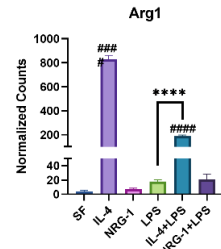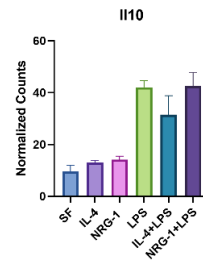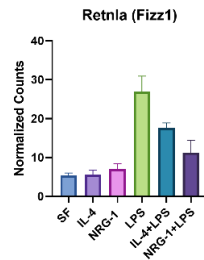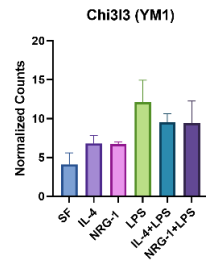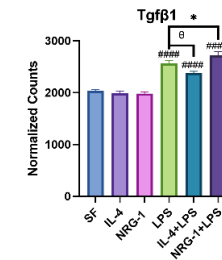
Figure 3-6 NRG-1 does not induce expression of traditional M2 factors

Bar charts show normalized mRNA count number of M2 markers over six chemical induction conditions. Top two rows show M2 markers that behaved as expected, with large increase following IL-4 exposure. Black box indicates M2 markers that were only upregulated by IL-4 in the presence of pro-inflammatory LPS stimulus. Row 3 contains M2 markers with unexpected expression outcomes. These include traditional M2 factors that were below detection (Chi3l3, Retnla, IL-10). Finally Tgfβ1 was increased by NRG-1 but not IL-4. NRG-1 generally did not induce increased gene expression of M2 factors, with the exception of Mrc1 and Ccl17, in which both IL-4 and NRG-1 showed significant gene expression increase over LPS alone, with the magnitude of NRG-1 effect being smaller than that achieved my IL-4. P-value: # baseline SF, *baseline LPS * = <0.05, **= <0.01, ***= <0.001, ****=<.0001, ns = not significant.

Within this cell model, we do not see strong expression of very many typical anti-inflammatory markers when we deliver IL-4 to SIM-A9 cells. However, the anti-inflammatory character of IL-4 at this dosage and in this system was evident in the survey of M1 factors. Therefore, we must conclude that IL-4 is behaving atypically in our context. However, it clearly has some activity. Therefore, the question becomes, what does the nature of anti-inflammatory stimulation look like in this cellular system? To answer this, we look at what factors are most effected by IL-4.

Taken together, NRG-1 treatment in a naïve context shows minimal activation of inflammatory pathways while acting as a repressor of inflammation when delivered within the pro-inflammatory context such as LPS activation.

CONCLUSION AIM 2A

From these experiments, it is clear that NRG-1 is able to induce changes within inflammatory cells. While some of these changes are the same as those induced in anti-inflammatory activation, within the NanoString panel consisting of important mediators of inflammation, NRG-1 shows aspects of both pro-inflammatory and anti-inflammatory gene expression, as well as unique effects. This suggests that NRG-1 may not be anti-inflammatory with respect to the canonical gene library. However previous *in vivo* work establishes that NRG-1 is an effective treatment for reducing neuroinflammation in rodent models of MCAO and nerve agent exposure(Xu,

Jiang et al. 2004, Xu, Ford et al. 2005, Li, Lein et al. 2015). Therefore, this

suggests that NRG-1 is immuno-modulatory and may have a role in other

regulatory pathways that were not well represented on this panel.

Our initial hypothesis that NRG-1 achieves anti-inflammatory effects by

reducing a portion of the LPS-induced response was not strongly supported

in this analysis of 248 genes within the inflammatory cytokine signaling

cascade. NRG-1 does not result in activation of inflammation when delivered

individually, in contrast to both the pro-inflammatory LPS and anti-

inflammatory IL-4 stimuli, which are both known to activate inflammation.

When NRG-1 is given concurrently with pro-inflammatory LPS stimulation did

not demonstrate a strong ability to change levels of inflammatory cytokines.

Concurrent treatment with anti-inflammatory IL-4 and pro-inflammatory LPS

resulted in far more changes in gene expression. The IL-4 response to LPS

stimulation is consistent with the current understanding of the anti-

inflammatory mechanism working by shutting off, reversing, or counteracting

pro-inflammatory cascades (Woodward, Prele et al. 2010). While NRG-1

showed some overlap with anti-inflammatory treatment IL-4 in its effect on

LPS-induced gene expression changes, these were generally lesser in

magnitude and involved a much smaller subset of genes. In addition, within

individual stimulation conditions, NRG-1 regulated some genes in the

opposing direction to both pro- and anti-inflammatory stimulations. To explore

the hypothesis that NRG-1 works primarily though other pathways outside of

inflammatory cytokine cascades, we expanded the number of genes

investigated from the 248 inflammatory genes used in the previous section to

a panel of 757 genes picked for importance in neuroinflammatory pathways.

CHAPTER 4 AIM 2B- EVALUATING THE EFFECT OF NRG-1 STIMULATION ON PRO-INFLAMMATRY GENE EXPRESSION CHANGES WITHIN NEUROPATHOLOGICAL PATHWAYS IN SIM-A9 MICROGLIA

ABSTRACT

Studies in Chapter 4 extend this work beyond inflammatory genes into

neuropathology (Neuropathology Panel,757 genes) widening the scope of

gene expression data to additional pathways associated with

neuroinflammation and neuropathology.

INTRODUCTION

These studies used the same culture conditions and general experimental

setup and samples isolation as outlined in Chapter 3 and used for

inflammation panel (see Chapter 3 sections Methods and Gene Expression

Analysis). The only change made to experimental procedure was that we

used a dose of 10ng/mL for NRG-1 as opposed to the 100 ng/mL used in the

inflammatory panel.

We refined the dosing, exploring efficacy of lower doses. After further

optimizing for the effective dose of NRG-1 in SIM-A9 cells, we found that a

dose of 10ng/mL NRG-1 produced strong reduction in LPS-induced

expression of specific markers NO and IL-6 (Appendix Fig-2.2). Lower doses

are advantageous for drug development to avoid toxicity and unforeseen

effects. Each treatment group had n=2 biological replicates.

METHODS

Experimental Design

Table 4-1
Exposure Conditions used in Neuropathology Panel

Stimulus	Dose
Serum Free	
IL-4	50 ng/mL
LPS	1 µg/mL
NRG-1	10 ng/mL
LPS + IL-4	1 µg/mL LPS + 50 ng/mL IL-4
LPS + NRG-1	1 µg/mL LPS + 10 ng/mL NRG-1

Panel Expression and Thresholding

# Genes	Neuropathology Panel
Total	757
Above Cutoff	565
Removed	192

Differential expression between treatments was calculated using the same protocol established in Aim 2A (Chapter 3) Differential gene expression was calculated with the Advanced Analysis plugin for nSolver DE module (NanoString MAN-10030-03) which uses an algorithm based upon a negative binomial model which changes based upon the magnitude of the counts. The algorithm has more power to discriminate between means for lower expression genes.

The Neuropathology panel consists of 757 genes known to be important in neuropathological pathways. These genes are spread over three main themes:

1. Immunity and Inflammation

2. Neurobiology and Neuropathology

3. Metabolism and Stress

Of these 757 genes, 192 were removed for being below the threshold of ≥20 counts for at least 10% of the samples, leaving 565 genes for further analysis. Using the serum-free condition as a baseline, we assessed global gene expression changes from baseline resulting from our three treatments of interest, LPS alone, IL-4 along, NRG-1 alone, LPS + IL-4, and LPS + NRG-1.

Overexpression or under-expression of themes and gene sets calculation is based upon the percentage of genes present in the sample, so it is necessary to determine how many genes are above the thresholding cutoff.

Themes

Table 4-2
Coverage of Neuropathology Panel Genes Expressed by Theme

	Total Genes (#)	Genes Above Threshold	% Above Threshold
Immunity and Inflammation	431	344	80%
Neurobiology and Neuropathology	507	387	76%
Metabolism and Stress	171	151	88%

Gene Sets

Table 4-3
Coverage of Neuropathology Panel Genes Expressed by Gene Set

Gene Set	Genes in Panel	% of Panel	Expressed Genes	% of Panel Expressed
Adaptive Immune Response	132	17%	103	18%
Angiogenesis	41	5%	33	6%
Apoptosis	140	18%	119	21%
Astrocyte Function	55	7%	29	5%
Autophagy	99	13%	90	16%
Carbohydrate Metabolism	10	1%	9	2%
Cell Cycle	69	9%	65	12%
Cellular Stress	81	11%	72	13%
Cytokine Signaling	117	15%	99	18%
DNA Damage	82	11%	77	14%
Epigenetic Regulation	70	9%	62	11%
Growth Factor Signaling	153	20%	130	23%
Inflammatory Signaling	103	14%	94	17%
Innate Immune Response	146	19%	126	22%
Insulin Signaling	30	4%	26	5%
Lipid Metabolism	18	2%	16	3%
Matrix Remodeling	44	6%	26	5%
Microglia Function	187	25%	154	27%
NF-kB	80	11%	53	9%
Neurons and Neurotransmission	60	8%	45	8%
Notch	23	3%	21	4%
Oligodendrocyte Function	27	4%	11	2%
Wnt	29	4%	26	5%

Pathway Analysis using Single and Combination Treatment Gene

Expression Changes

Gene expression changes were calculated as in Chapter 3.

PANTHER Methods

PANTHER overexpression analysis gives an enrichment score (expected # genes/#number in your dataset) and p-value. It also shown the number of genes in the set or pathway.

Used binomial test and Bonferroni correction and considered all p-values less than 0.5 to be significant. Used enrichment score as the metric to order the GO terms.

Triaging criteria

Searched for enrichment of GO terms and pathways from reactome and PANTHER pathways.

For all significant GO terms or Pathways, top paths are ordered by enrichment score

Gene expressions were uploaded to PANTHER to determine enriched GO terms and pathways. Resulting pathways diagrams were selected from KEGG database and colorized using KEGG web applet (citation).

Analysis of Regulation of Gene Sets

For genes expressed in each gene set, we preformed simple enrichment analysis. For simple enrichment analysis a gene set was defined as enriched for a particular treatment if treatment-induced gene expression changes for a gene set was greater than the percentage of the total panel that gene set represented. A gene set was considered well-represented if at least half of the genes contained on the pathway were present.

RESULTS AND DISCUSSION

Changes in Neuropathology Panel Gene Expression Following Individual Treatment Induction with LPS, IL-4 or NRG-1

As in Chapter 3, we tracked how gene expression changed following individual treatments with induction factors (Table 4-4). In corroboration with our observations in Aim 2A, NRG-1 treatment alone resulted in very few changes. The majority, 34 out of 37 genes or 92%, of changes NRG-1 does cause are upregulations from baseline. This is in contrast to both pro-inflammatory LPS and anti-inflammatory IL-4, both of which cause an approximately even split between upregulations and downregulations.

Table 4-4 Gene Expression Changes in Neuropathology Panel Relative to Baseline of Unstimulated Cells

Differentially Regulated genes	LPS	IL-4	NRG-1	LPS + IL-4	LPS + NRG-1
Total	315	131	37	305	314
Upregulated (%)	153 (49%)	79 (60%)	34 (92%)	160 (52%)	172 (55%)
Downregulated (%)	162 (51%)	52 (40%)	3 (8%)	145 (48%)	142 (45%)

In Tables 4-5 and 4-6 the regulation of significantly changed genes following individual exposure to NRG-1 are outlined in comparison to individual stimulation with either pro-inflammatory LPS or anti-inflammatory IL-4. In contrast to what we saw for the inflammation panel in chapter 3, for genes within the neuropathology panel show more overlap with the LPS-induced response than the IL-4 induced response.

Table 4-5

Gene Expression Changes Resulting From Stimulation with Individual Induction Factor Treatments which are not Significantly Regulated in all Three Inductions

NRG (8)		NRG-1 and LPS (9)				NRG -1 and IL-4 (5)			
		Same (8)		Opposite (1)		Same (3)		Opposite (2)	
↑	↓	↑	↓	↑	↓	↑	↓	↑	↓
8	0	6	2	1		3			2
Ccng2		Cd68	Fancd2	Fancg		Brd4		Cd33	
Dot1l		Cdkn1a	Ung			Fgf13		Tpd52	
Igsf10		Hmox1				Rab6b			
Kmt2c		Ikbkb							
Mbd2		Mdm2							
P2ry12		Pla2g5							
Pik3r5									
Rps21									

Key: When sig. for LPS and NRG-1 only: ■ Regulated same between LPS and NRG-1; ■ Regulated oppositely between LPS and NRG-1
When sig. for LPS and NRG-1 only: ■ Regulated same between IL-4; ■ Regulated oppositely between IL-4 and NRG-1
When significant for NRG-1 ONLY: ■ Upregulated ■ Downregulated by NRG-1

Table 4-6

Genes with Significant Expression Changes in all Individual Stumulus Conditions

All (15)							
All Same direction (7)		NRG unique (1)		NRG like LPS (6)		NRG like IL-4 (1)	
↑	↓	↑	↓	↑	↓	↑	↓
7			1	6			1
Cd40			Ccl5	Hilpda			Sin3a
Cd47				Il21r			
Cd83				Lcn2			
Ddx58				Prdx1			
Ifih1				Slc2a1			
Lrrc25				Srgn			
Sod2							

Key: ■ Regulated in same direction for all conditions; ■ Regulated same between LPS and NRG; ■ Regulated same between IL-4; ■ Unique Regulation by NRG-1
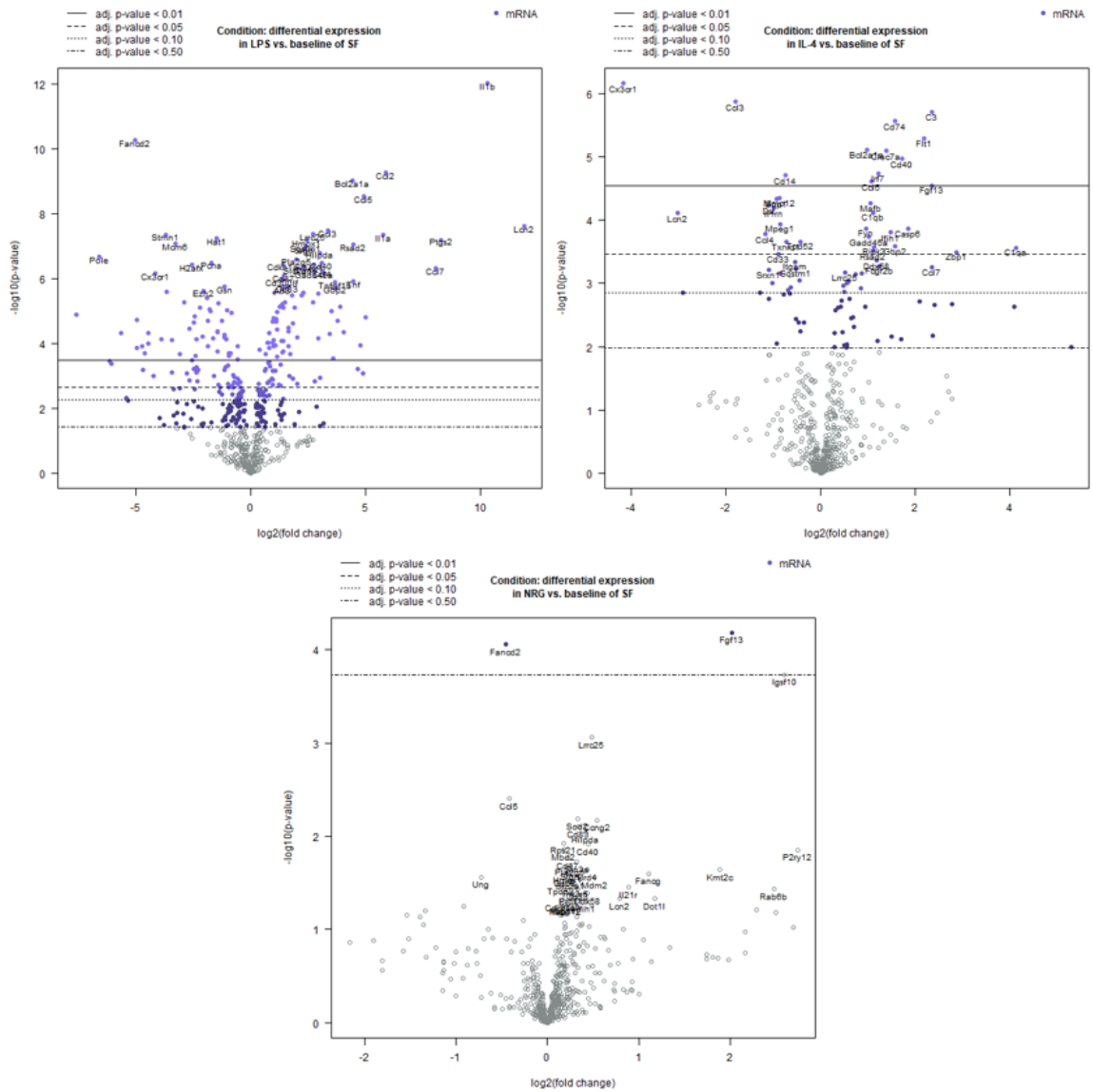
Figure 4-1 Volcano plots
Dataset for Neuroinflammatory panel in NanoString. Top 20 significant genes labeled on graph. Colored circle indicates significant difference.

Volcano plots (Figure 4-1) of exposure of unstimulated cells with individual

induction factors show that NRG-1 results in a much narrower range of

expression changes. These findings provide further evidence that NRG-1

delivered individually does not activate microglia.

Table 4-7
Differentially Expressed Genes that are Significant in NRG-1 Only

	LPS	IL4	NRG-1
Ccng2			+
Dot1l			++
Igsf10			+++
Kmt2c			++
Mbd2			+
P2ry12			+++
Pik3r5			+

+ $\log_2$ fold change between 0 and 1
++ $\log_2$ fold change between 1 and 2
+++ $\log_2$ fold change greater than 2

Eight (8) genes were significant in only NRG-1 (Table 4-7

Differentially Expressed Genes that are Significant in NRG-1 Only. All eight

genes were upregulated by NRG-1 with 4 of being upregulated at least 2-fold

but only Igsf10 and P2ry12 showed a greater than 2-fold **Error! Reference**

source not found.difference (which is a common cutoff looking at gene

expression changes).

Table 4-8
NRG-1 Treatment Regulates Genes in Opposition to Inflammatory
Activators

	LPS	IL4	NRG-1
Ccl5	+++	++	-
Cd33		-	+
Fancg	--		++
Tpd52		-	+

+ $\log_2$ fold change between 0 and 1
++ $\log_2$ fold change between 1 and 2
+++ $\log_2$ fold change greater than 2

In addition, four genes were uniquely regulated in the opposing direction in

NRG-1 treatment as compared to both the known treatments (**Error!**

Reference source not found.). NRG-1 uniquely downregulated CCL5 which

was upregulated in both the pro-inflammatory and anti-inflammatory conditions. In CD33, NRG-1 upregulated expression while both inflammatory molecules showed downregulation of this gene (although downregulation was only significant in IL-4; In LPS the downregulation was a trend). Tdp52 showed a similar pattern of expression as CD33. Finally, Fancg showed one of the clearest differences, as it was 2-fold upregulated in NRG-1 while 2-fold downregulated in LPS. There is evidence that this may be behavior more indicative of the anti-inflammatory behavior as the nonsignificant trend in IL-4 was also towards upregulation. The other three genes, CD33, Fancg, and Tpd52, were upregulated in but none of these expression changes was greater than 2-fold.

Tpd52 is a cell surface marker that is expressed when microglia are in physical contact with endothelial cells. It has also been identified as a marker in prostate cancer and as a part of VGLUT1 containing synapses.

Gene for which NRG-1 resulted in regulation in the same direction as either LPS or IL-4 are shown in Table 4-9. Although twice the number of genes are regulated similarly between LPS and NRG-1 as to between IL-4 and NRG-1, the number of genes changed by NRG-1 is MUCH smaller, therefore, it would not be accurate to conclude that NRG-1 behaves like LPS. Rather, we can see that NRG-1 is unlike LPS or IL-4 stimulation.

Flt1, also known as VGFR1, a receptor for adhesive molecules is downregulated according to the neuroinflammation panel from NanoString,

and minimally increased according to inflammation NanoString. IL-4 has a strong inducing effect. Expression of this receptor plays a role in microglial chemotaxis and has been shown to be pathological in Alzheimer's.

Table 4-9
NRG-1 Causes Similar Gene Expression Change as Known Inflammatory Activators

	LPS	IL4	NRG-1
Brd4		+	+
Cd40	+++	++	+
Cd47	++	+	+
Cd68	+		+
Cd83	++	+	+
Cdkn1a	++		+
Ddx58	++	++	+
Fancd2	---		-
Fgf13		+++	+++
Hilpda	+++	-	+
Hmox1	+++		+
Ifih1	+++	++	+
Ikbkb	+		+
Il21r	++	-	+
Lcn2	+++	---	+
Lrrc25	+++	+	+
Mdm2	+		+
Pla2g5	++		+
Prdx1	+	-	+
Rab6b		+++	+++
Sin3a	-	+	+
Slc2a1	+++	-	+
Sod2	+	+	+
Srgn	+++	-	+
Tpd52		-	+
Ung	--		-

Green – NRG-1 behaves like IL-4
Light green – not significant difference for LPS
Dark green – LPS regulated oppositely
Grey – All treatments cause same direction of regulation
Red

NRG-1 Regulation of Gene Expression Changes Following Combined

Stimulation NRG-1 and LPS of SIM-A9 Cells

As in Chapter 3, combination treatments are compared against a baseline of

LPS single treatment rather than unstimulated cells in order to isolate gene

expression changes caused only by the influence of the combined treatment

factor, either NRG-1 or IL-4. Gene expression changes are outlined in Table

4-11 and Table 4-12.

Table 4-10
Stimulus-Induced Neuropathology Gene Expression Changes

Baseline	LPS	IL-4+LPS	NRG-1+LPS
Unstimulated (Serum Free)	315	305	314
LPS	--	139	89

We can see that LPS + NRG-1 induced changes from LPS-induced single

treatment gene expression that were spread almost equally among reversed,

enhanced, and unique regulation while LPS + IL-4 combined treatment gene

expression changes overwhelmingly led to reversed regulation. Combined

LPS + NRG-1 treatment resulted in a total of 90 gene expression changes,

70 upregulations and 20 downregulations as compared to LPS alone.
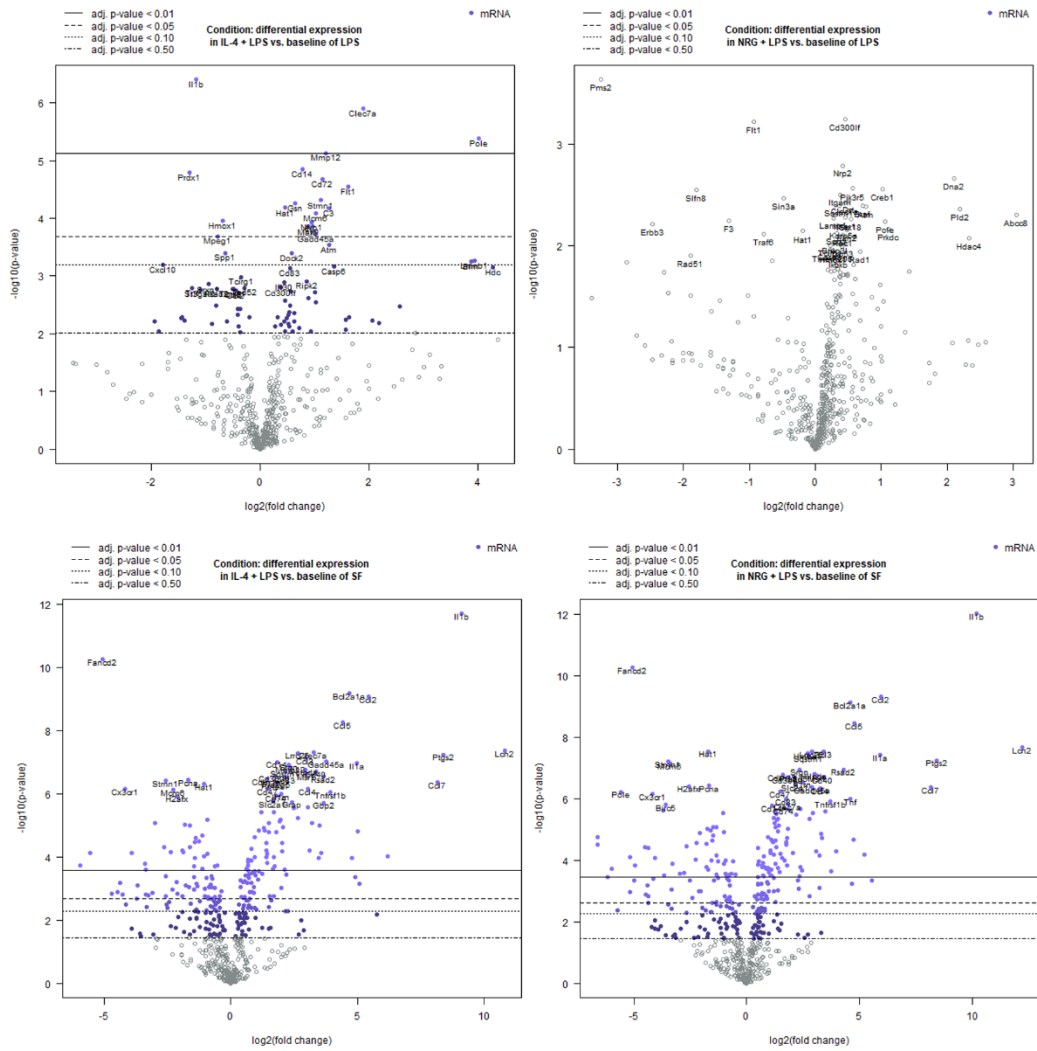
Figure 4-2 Volcano Plots for Combination Stimulations

LPS baseline allows clearer visualization of the differences between the influence of NRG-1 and IL-4 on inflammation for Neuropathology gene expression. Upper panels show volcano plots with LPS baseline. Lower panels show plots with unstimulated serum free baseline.

Table 4-11
Total Gene Expression Changes induced by Combination Treatments

		Total				Shared	
		NRG-1		IL-4		Shared	
Reverse	Up	31	28	91	56	14	13
	Down		3		35		1
Enhance	Up	34	25	21	18	7	6
	Down		9		3		1
Unique	Up	25	17	27	14	4	2
	Down		8		13		2

Table 4-12
Stimulus-Specific and Shared Gene Expression Changes induced by Combination Treatments

		Unique Response				Shared	
		NRG-1		IL-4		Shared	
Reverse	Up	17	15	77	43	14	13
	Down		2		34		1
Enhance	Up	27	19	14	12	7	6
	Down		8		2		1
Unique	Up	21	15	23	12	4	2
	Down		6		11		2
Total		65		114		25	

NRG-1 and LPS Combined Stimulation Causes Relatively Fewer Reversals of

LPS-Induced Gene Expression Changes

Table 4-11 (Top Lines) shows that combination LPS + NRG-1 was shown to

reverse many gene expression changes, but far fewer than combination LPS

+ IL-4 and about half of the changes were shared between both treatments.

These reversed genes are listed in Table 4-13.

Table 4-13
Addition of NRG-1 Reverses LPS-Induced Gene Expression Changes in Neuropathology Panel

Gene Symbol	Gene Name	Fold Change	
		NRG-1+ LPS	IL-4+ LPS
Bnip3l	BCL2/adenovirus E1B interacting protein 3-like	1.23	1.12
Dapk1	Death associated protein kinase 1	1.31	1.17
Dna2	DNA replication helicase/nuclease 2	4.28	1.00
Hdac2	Histone deacetylase 2	1.41	-1.02
Hdac4	Histone deacetylase 4	5.02	-1.73
Lamp1	Lysosomal-associated membrane protein 1	1.21	-1.06
Lgmn	Legumain	1.22	1.15
Man2b1	Mannosidase 2, alpha B1	1.54	-1.04
Msh2	MutS homolog 2	1.92	1.84
Prkdc	Protein kinase, DNA activated, catalytic polypeptide	2.15	1.31
Rac1	RAS-related C3 botulinum substrate 1	1.33	-1.05
Ralb	V-ral simian leukemia viral oncogene B	1.30	1.20
Rps2	Ribosomal protein S2	1.15	1.13
Tmem64	Transmembrane protein 64	1.24	-1.13
Trp53	Transformation related protein 53	1.32	1.08
F3	Coagulation factor III	-2.48	-1.61
Hdc	Histidine decarboxylase	-3.72	19.50
Abcc8	**ATP-binding cassette, sub-family C (CFTR/MRP), member 8**	8.20	4.00
Atm	Ataxia telangiectasia mutated	1.69	2.42
Atr	Ataxia telangiectasia and Rad3 related	2.04	3.09
C1qc	Complement component 1, q subcomponent, C chain	1.81	2.05
Creb1	**CAMP responsive element binding protein 1**	2.03	2.02
Crebbp	**CREB binding protein**	1.29	1.26
Ezh2	**Enhancer of zeste 2 polycomb repressive complex 2 subunit**	1.39	1.37
Ifnar1	**Interferon (alpha and beta) receptor 1**	1.37	1.35
Pld2	**Phospholipase D2**	4.56	2.72
Pole	Polymerase (DNA directed), epsilon	2.09	16.10
Ptms	Parathymosin	1.33	1.44
Rad1	**RAD1 checkpoint DNA exonuclease**	1.60	1.55
Tmem206	Transmembrane protein 206	1.20	1.20
Erbb3	**Erb-b2 receptor tyrosine kinase 3**	-5.56	-3.22

Genes above the double line significant in NRG-1 treatment only
Green indicates upregulation compared to a baseline of LPS; Red indicates downregulation compared to a baseline of LPS; Grey indicates p-value for difference is not significant
Bold denotes genes where NRG-1 treatment resulted in a greater magnitude gene expression change than IL-4

The Most Common Regulation by Combined Stimulation with NRG-1 and LPS was Enhancement of LPS-induced Expression

Combined LPS + NRG-1 treatment resulted nearly double the number of enhanced genes than combined LPS + IL-4 treatment (27 and 14 genes respectively, Table 4-14) and only 20% of the total enhancement caused by NRG-1 + LPS was shared with IL-4 + LPS. This is the only one of the three regulation types (reversed, enhanced, unique) in which NRG-1 affected a greater number of genes than IL-4. IL-4 is known to be anti-inflammatory in part by reversing pro-inflammatory induced changes. However, a change towards anti-inflammatory activation is not always beneficial. Many infections evade the immune system by initiating anti-inflammatory activation, which does not effectively clear the infection or the damaged tissues. Another way to conceptualize this would be to assume that cells generally evolve to react in ways that tend to lead to a favorable outcome for the organism. Therefore, in response to pro-inflammatory stimuli, it is likely to be in the cell's best interests to respond with pro-inflammatory activation. To act as an effective treatment aid, treatment may be most effective when it enhances the cell's natural response. Such regulation would show up as an enhanced gene expression change in this analysis.

This suggests that a key part of the mechanism NRG-1 uses as an effective

treatment may be to strengthen some aspects of the pro-inflammatory

response. This may allow the pro-inflammatory phase of response to be

more effective or shorter-lived, as many pro-inflammatory cycles self-regulate

through negative feedback loops.

Table 4-14

Neuropathology Panel Genes where Combined Treatment with NRG-1 Enhanced LPS-induced Gene Expression Changes

Gene Symbol	Gene Name	Fold Change	
		NRG-1+ LPS	IL-4+ LPS
Asph	Aspartate-beta-hydroxylase	1.18	1.01
Birc3	Baculoviral IAP repeat-containing 3	1.51	1.25
Casp8	Caspase 8	1.17	1.03
Cdkn1a	Cyclin-dependent kinase inhibitor 1A (P21)	1.14	1.01
Dst	Dystonin	1.42	-1.23
Hmox1	Heme oxygenase 1	1.21	-1.60
Ikbkb	Inhibitor of kappaB kinase beta	1.28	1.10
Il21r	Interleukin 21 receptor	1.97	1.17
Itgam	Integrin alpha M	1.30	-1.01
Ly9	Lymphocyte antigen 9	1.35	1.02
Msn	Moesin	1.30	-1.03
Msr1	Macrophage scavenger receptor 1	1.29	1.17
Nfkb2	Nuclear factor of kappa light polypeptide gene enhancer in B cells 2, p49/p100	1.38	1.23
Nrp2	Neuropilin 2	1.33	-1.09
Sod2	Superoxide dismutase 2, mitochondrial	1.25	1.12
Spp1	Secreted phosphoprotein 1	1.22	-1.55
Sqstm1	Sequestosome 1	1.29	-1.19
Tcirg1	T cell, immune regulator 1, ATPase, H+ transporting, lysosomal V0 protein A3	1.17	-1.28
Txnrd1	Thioredoxin reductase 1	1.24	-1.41
Axl	AXL receptor tyrosine kinase	-2.03	-1.20
Birc5	Baculoviral IAP repeat-containing 5	-1.58	2.01
Hat1	Histone aminotransferase 1	-1.14	1.37
Pms2	PMS1 homolog2, mismatch repair system component	-9.52	1.18
Rad51	RAD51 recombinase	-3.70	4.58
Sin3a	Transcriptional regulator, SIN3A (yeast)	-1.39	1.26
Suv39h1	Suppressor of variegation 3-9 1	-1.90	1.20
Bcl2a1a	B cell leukemia/lymphoma 2 related protein A1a	1.13	1.21
Cd300lf	**CD300 molecule like family member F**	1.37	1.30
Cd74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	1.22	1.37
Clec7a	C-type lectin domain family 7, member a	1.37	3.72
Itgav	Integrin alpha V	1.26	1.44
Stx18	**Syntaxin 18**	1.45	1.43
Chek1	**Checkpoint kinase 1**	-4.69	-4.48

Genes above the double line significant in NRG-1 treatment only

Green indicates upregulation compared to a baseline of LPS; Red indicates downregulation compared to a baseline of LPS; Grey indicates p-value for difference is not significant

Bold denotes genes where NRG-1 treatment resulted in a greater magnitude gene expression change than IL-4

<u>Unique Changes in Gene Expression as a Result of Combined Stimulation with</u>

<u>LPS and NRG-1</u>

Three of the 25 unique changes were also significantly different for individual

NRG- 1 stimulation of unstimulated cells, highlighting their importance in

NRG-1 induced responses (Table 4-15). Kmt2c and Pik3r5 were unique

differences induced by NRG-1 and Cd33 was uniquely upregulated by NRG-

1 in contrast to IL-4 where CD33 was significant but downregulated. For

Pik3r5 and Cd33, unique regulation is in the same direction as individual

regulation while Kmt2c is upregulated when delivered individually but

downregulated uniquely. Finally, only Cd33 was uniquely upregulated in

combined and individual excitation with NRG-1, indicating it may be a

potential marker for NRG-1 stimulation. Pik3r5 is also a potential marker of

NRG-1 stimulus response, but it is not unique as it is shared with IL-4

response. The change in regulation between individual and combined

stimulation for Kmt2c.

Table 4-15
Combination LPS + NRG-1 Uniquely Changes Gene Expression

Gene Symbol	Gene Name	Fold Change	
		NRG-1+ LPS	IL-4+ LPS
Bin1	Bridging integrator 1	1.27	1.06
Braf	Braf transforming gene	1.64	1.15
Cd33	CD33 antigen	1.27	-1.03
Chuk	Conserved helix-loop-helix ubiquitous kinase	1.24	1.14
Csf1	Colony stimulating factor 1 (macrophage)	1.25	-1.17
Eed	Embryonic ectoderm development	1.30	1.18
Igf2r	Insulin-like growth factor 2 receptor	1.40	1.01
Kdm5a	Lysine (K)-specific demethylase 5A	1.35	-1.04
Kmt2a	Lysine (K)-specific methyltransferase 2A	1.48	-1.32
Lst1	Leukocyte specific transcript 1	2.68	1.16
Psen2	Presenilin 2	1.42	1.02
Rela	V-rel reticuloendotheliosis viral oncogene homolog A (avian)	1.19	-1.04
Ripk1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	1.49	1.13
Rpl28	Ribosomal protein L28	1.20	1.09
Tfg	Trk-fused gene	1.30	1.06
Fas	Fas (TNF receptor superfamily member 6)	-2.74	-2.26
Flt1	FMS-like tyrosine kinase 1	-1.91	3.07
Kmt2c	Lysine (K)-specific methyltransferase 2C	-2.97	1.19
Nbn	Nibrin	-4.88	-2.25
Slfn8	Schlafen 8	-3.47	-1.37
Traf6	TNF receptor-associated factor 6	-1.72	-1.05
Fabp5	Fatty acid binding protein 5, epidermal	1.18	1.24
Pik3r5	**Phosphoinositide-3-kinase regulatory subunit 5**	1.48	1.46
Fgd2	FYVE, RhoGEF and PH domain containing 2	-10.50	-10.50
Foxp3	**Forkhead box P3**	-7.25	-3.23

Genes above the double line significant in NRG-1 treatment only
Green indicates upregulation compared to a baseline of LPS; Red indicates downregulation compared to a baseline of LPS; Grey indicates p-value for difference is not significant
Bold denotes genes where NRG-1 treatment resulted in a greater magnitude gene expression change than IL-4

Gene Set Analysis of NRG-1 Effects on Neuropathological Gene Expression

Table 4-16 shows regulation of gene sets. These numbers are all in

reference to a baseline of serum-free media.

The only gene set for which NRG-1+ LPS treatment shows any uniquely

enriched gene expression is within the set of genes for apoptosis. The

apoptosis gene set includes genes from many pathways, but one of the

major contributors is the NF-kB pathway, which was also identified as an

important pathway in our previous analysis.

Table 4-16

Enrichment Scores for Gene Sets within the Neuropathology Theme

Gene Sets		LPS	IL-4 + LPS	NRG + LPS	IL-4	NRG-1
Angiogenesis	Total (% of regulated genes)	24 (8%)	20 (7%)	20 (6%)	6 (5%)	0 (0%)
	Gene Set Coverage	59%	49%	49%	15%	0%
	↑ (% genes in gene set)	11 (46%)	11 (55%)	11 (55%)	5 (83%)	0 (%)
	↓ (% genes in gene set)	13 (54%)	9 (45%)	9 (45%)	1 (17%)	0 (%)
Apoptosis	Total (% of regulated genes)	64 (20%)	61 (20%)	70 (22%)	23 (18%)	4 (11%)
	Gene Set Coverage	46%	44%	50%	16%	3%
	↑ (% genes in gene set)	33 (52%)	36 (59%)	42 (60%)	16 (70%)	4 (100%)
	↓ (% genes in gene set)	31 (48%)	25 (41%)	28 (40%)	7 (30%)	0 (0%)
Astrocyte Function	Total (% of regulated genes)	19 (6%)	16 (5%)	20 (6%)	11 (8%)	2 (5%)
	Gene Set Coverage	35%	29%	36%	20%	4%
	↑ (% genes in gene set)	16 (84%)	14 (88%)	16 (80%)	7 (64%)	2 (100%)
	↓ (% genes in gene set)	3 (16%)	2 (13%)	4 (20%)	4 (36%)	0 (0%)

Gene Sets		LPS	IL-4 + LPS	NRG + LPS	IL-4	NRG-1
Cell Cycle	Total (% of regulated genes)	49 (16%)	45 (15%)	47 (15%)	6 (5%)	3 (8%)
	Gene Set Coverage	71%	65%	68%	9%	4%
	↑ (% genes in gene set)	7 (14%)	7 (16%)	7 (15%)	3 (50%)	3 (100%)
	↓ (% genes in gene set)	42 (86%)	38 (84%)	40 (85%)	3 (50%)	0 (0%)
DNA Damage	Total (% of regulated genes)	52 (17%)	45 (15%)	52 (17%)	4 (3%)	6 (16%)
	Gene Set Coverage	63%	55%	63%	5%	5%
	↑ (% genes in gene set)	7 (13%)	7 (16%)	7 (13%)	3 (75%)	4 (67%)
	↓ (% genes in gene set)	45 (87%)	38 (84%)	45 (87%)	1 (25%)	2 (33%)
Epigenetic Regulation	Total (% of regulated genes)	29 (9%)	32 (10%)	25 (8%)	6 (5%)	5 (14%)
	Gene Set Coverage	41%	46%	36%	9%	7%
	↑ (% genes in gene set)	4 (14%)	6 (19%)	5 (20%)	6 (100%)	5 (100%)
	↓ (% genes in gene set)	25 (86%)	26 (81%)	20 (80%)	0 (0%)	0 (0%)

Gene Sets		LPS	IL-4 + LPS	NRG + LPS	IL-4	NRG-1
Growth Factor Signaling	Total (% of regulated genes)	79 (25%)	76 (25%)	79 (25%)	29 (22%)	6 (16%)
	Gene Set Coverage	52%	50%	52%	19%	4%
	↑ (% genes in gene set)	39 (49%)	43 (57%)	47 (59%)	16 (55%)	6 (100%)
	↓ (% genes in gene set)	40 (51%)	33 (43%)	32 (41%)	13 (45%)	0 (0%)
Insulin Signaling	Total (% of regulated genes)	14 (4%)	8 (3%)	11 (4%)	7 (5%)	0 (0%)
	Gene Set Coverage	47%	27%	37%	23%	0%
	↑ (% genes in gene set)	7 (50%)	6 (75%)	7 (64%)	5 (71%)	0 (%)
	↓ (% genes in gene set)	7 (50%)	2 (25%)	4 (36%)	2 (29%)	0 (%)
Matrix Remodeling	Total (% of regulated genes)	18 (6%)	14 (5%)	15 (5%)	7 (5%)	2 (5%)
	Gene Set Coverage	41%	32%	34%	16%	5%
	↑ (% genes in gene set)	14 (78%)	12 (86%)	13 (87%)	3 (43%)	2 (100%)
	↓ (% genes in gene set)	4 (22%)	2 (14%)	2 (13%)	4 (57%)	0 (0%)

Gene Sets		LPS	IL-4 + LPS	NRG + LPS	IL-4	NRG-1
Neurons and Neurotransmission	Total (% of regulated genes)	30 (10%)	30 (10%)	34 (11%)	8 (6%)	3 (8%)
	Gene Set Coverage	50%	50%	57%	13%	5%
	↑ (% genes in gene set)	23 (77%)	24 (80%)	27 (79%)	5 (63%)	3 (100%)
	↓ (% genes in gene set)	7 (23%)	6 (20%)	7 (21%)	3 (38%)	0 (0%)
Notch	Total (% of regulated genes)	11 (3%)	8 (3%)	9 (3%)	2 (2%)	0 (0%)
	Gene Set Coverage	48%	35%	39%	9%	0%
	↑ (% genes in gene set)	2 (18%)	1 (13%)	4 (44%)	0 (0%)	0 (%)
	↓ (% genes in gene set)	9 (82%)	7 (88%)	5 (56%)	2 (100%)	0 (%)
Oligodendrocyte Function	Total (% of regulated genes)	2 (1%)	2 (1%)	2 (1%)	2 (2%)	0 (0%)
	Gene Set Coverage	7%	7%	7%	7%	0%
	↑ (% genes in gene set)	1 (50%)	1 (50%)	1 (50%)	2 (100%)	0 (%)
	↓ (% genes in gene set)	1 (50%)	1 (50%)	1 (50%)	0 (0%)	0 (%)

Gene Sets		LPS	IL-4 + LPS	NRG + LPS	IL-4	NRG-1
Wnt	Total (% of regulated genes)	16 (5%)	14 (5%)	14 (4%)	2 (2%)	0 (0%)
	Gene Set Coverage	55%	48%	48%	7%	0%
	↑ (% genes in gene set)	3 (19%)	4 (29%)	4 (29%)	1 (50%)	0 (%)
	↓ (% genes in gene set)	13 (81%)	10 (71%)	10 (71%)	1 (50%)	0 (%)

Table 4-17 Gene Sets for Immunology Theme

Gene Sets		LPS	IL-4 + LPS	NRG + LPS	IL-4	NRG-1
Adaptive Immune Response	Total (% of regulated genes)	67 (21%)	59 (19%)	63 (20%)	24 (18%)	6 (16%)
	Gene Set Coverage	51%	45%	48%	18%	5%
	↑ (% genes in gene set)	36 (54%)	36 (61%)	40 (63%)	18 (75%)	6 (100%)
	↓ (% genes in gene set)	31 (46%)	23 (39%)	23 (37%)	6 (25%)	0 (0%)
Cytokine Signaling	Total (% of regulated genes)	55 (17%)	52 (17%)	56 (18%)	29 (22%)	5 (14%)
	Gene Set Coverage	47%	44%	48%	25%	3%
	↑ (% genes in gene set)	40 (73%)	42 (81%)	45 (80%)	16 (55%)	4 (80%)
	↓ (% genes in gene set)	15 (27%)	10 (19%)	11 (20%)	13 (45%)	1 (20%)
Inflammatory Signaling	Total (% of regulated genes)	57 (18%)	52 (17%)	58 (18%)	33 (25%)	7 (19%)
	Gene Set Coverage	55%	50%	56%	32%	6%
	↑ (% genes in gene set)	43 (75%)	42 (81%)	47 (81%)	25 (76%)	6 (86%)
	↓ (% genes in gene set)	14 (25%)	10 (19%)	11 (19%)	8 (24%)	1 (14%)

Gene Sets		LPS	IL-4 + LPS	NRG + LPS	IL-4	NRG-1
Innate Immune Response	Total (% of regulated genes)	80 (25%)	74 (24%)	82 (26%)	33 (25%)	5 (14%)
	Gene Set Coverage	55%	51%	56%	23%	3%
	↑ (% genes in gene set)	46 (58%)	47 (64%)	54 (66%)	21 (64%)	4 (80%)
	↓ (% genes in gene set)	34 (43%)	27 (36%)	28 (34%)	12 (36%)	1 (20%)
Microglia Function	Total (% of regulated genes)	95 (30%)	96 (31%)	93 (30%)	54 (41%)	13 (35%)
	Gene Set Coverage	51%	51%	50%	29%	6%
	↑ (% genes in gene set)	53 (56%)	56 (58%)	59 (63%)	27 (50%)	12 (92%)
	↓ (% genes in gene set)	42 (44%)	40 (42%)	34 (37%)	27 (50%)	1 (8%)
NF-kB	Total (% of regulated genes)	19 (6%)	20 (7%)	21 (7%)	7 (5%)	2 (5%)
	Gene Set Coverage	24%	25%	26%	9%	3%
	↑ (% genes in gene set)	9 (47%)	11 (55%)	14 (67%)	4 (57%)	2 (100%)
	↓ (% genes in gene set)	10 (53%)	9 (45%)	7 (33%)	3 (43%)	0 (0%)

For the themes of inflammation and neurobiology, NRG-1 influence was able to change the regulation pattern of genes in the panel (Table 4-18). For both these themes, NRG-1 influence resulted in more upregulated genes and fewer downregulated genes than in LPS stimulation alone.

Table 4-18 Gene Expression Changes for Panel Themes

Themes		LPS	IL-4 + LPS	NRG + LPS	IL-4	NRG-1
Immunity and Inflammation	**Total** (% of regulated genes)	209 (66%)	197 (65%)	209 (67%)	105 (80%)	23 (62%)
	↑ (% genes in theme)	120 (57%)	124 (63%)	136 (65%)	61 (58%)	22 (96%)
	↓ (% genes in theme)	89 (43%)	73 (37%)	73 (35%)	44 (42%)	1 (4%)
Neurobiology and Neuropathology	**Total** (% of regulated genes)	214 (68%)	209 (69%)	216 (69%)	71 (54%)	20 (54%)
	↑ (% genes in theme)	87 (41%)	96 (46%)	102 (47%)	47 (66%)	18 (90%)
	↓ (% genes in theme)	127 (59%)	113 (54%)	114 (53%)	24 (34%)	2 (10%)
Metabolism and Stress	**Total** (% of regulated genes)	101(32%)	90 (30%)	94 (30%)	30 (23%)	8 (22%)
	↑ (% genes in theme)	55 (54%)	54 (60%)	58 (62%)	15 (50%)	7 (88%)
	↓ (% genes in theme)	46 (46%)	36 (40%)	36 (38%)	15 (50%)	1 (13%)

Looking at global significance scores from LPS alone compared to NRG-1+ LPS (both with respect to baseline, Figure 4-3), the eleven (11) most involved pathways are in the same rank order and are very similar in order of magnitude. The most differentially regulated pathways for LPS and NRG-1+

LPS are the same, and include NF-kB, cellular stress, inflammatory signaling, cytokine signaling, innate immune response and apoptosis. NRG-1 treatment caused a change in pathway involvement in nine pathways, with five increasing involvement with treatment and four showing less involvement than LPS alone. Checking directed pathways scores for these pathways (Figure 4-4), the five more involved pathways were all upregulated and the 4 less involved pathways were down regulated. NRG-1 treatment scores were closer to those of IL-4 treatment in the four upregulated pathways for adaptive immune response, angiogenesis, autophagy, and matrix remodeling, and the downregulated Notch pathway. In contrast, scores were more like LPS in the upregulated pathway astrocyte function and downregulated pathways for carbohydrate metabolism, oligodendrocyte function, and Wnt signaling. Taken together, these data suggest that NRG-1 increases activation of repair oriented and cell mobilization pathways, in a similar manner as anti-inflammatory treatment while removing deactivation that is induced by pro-inflammatory treatment.

Gene Sets
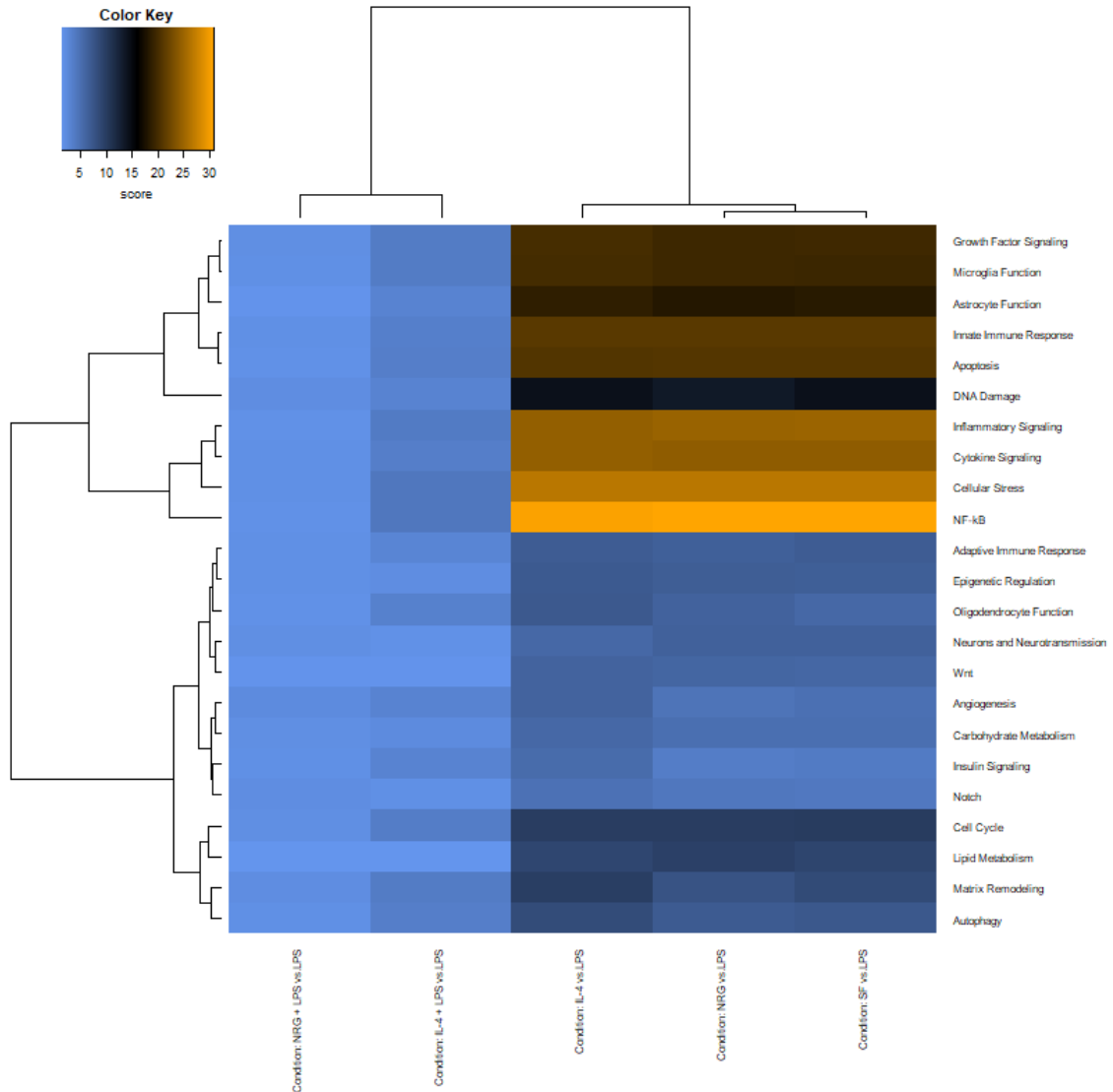
Figure 4-3: Hierarchical Clustered dendrogram of global significance scores.

Global significance calculates the total magnitude of change within a gene set. Hierarchical clustering shows Scores are calculated for each treatment relative to the baseline of serum free cells.
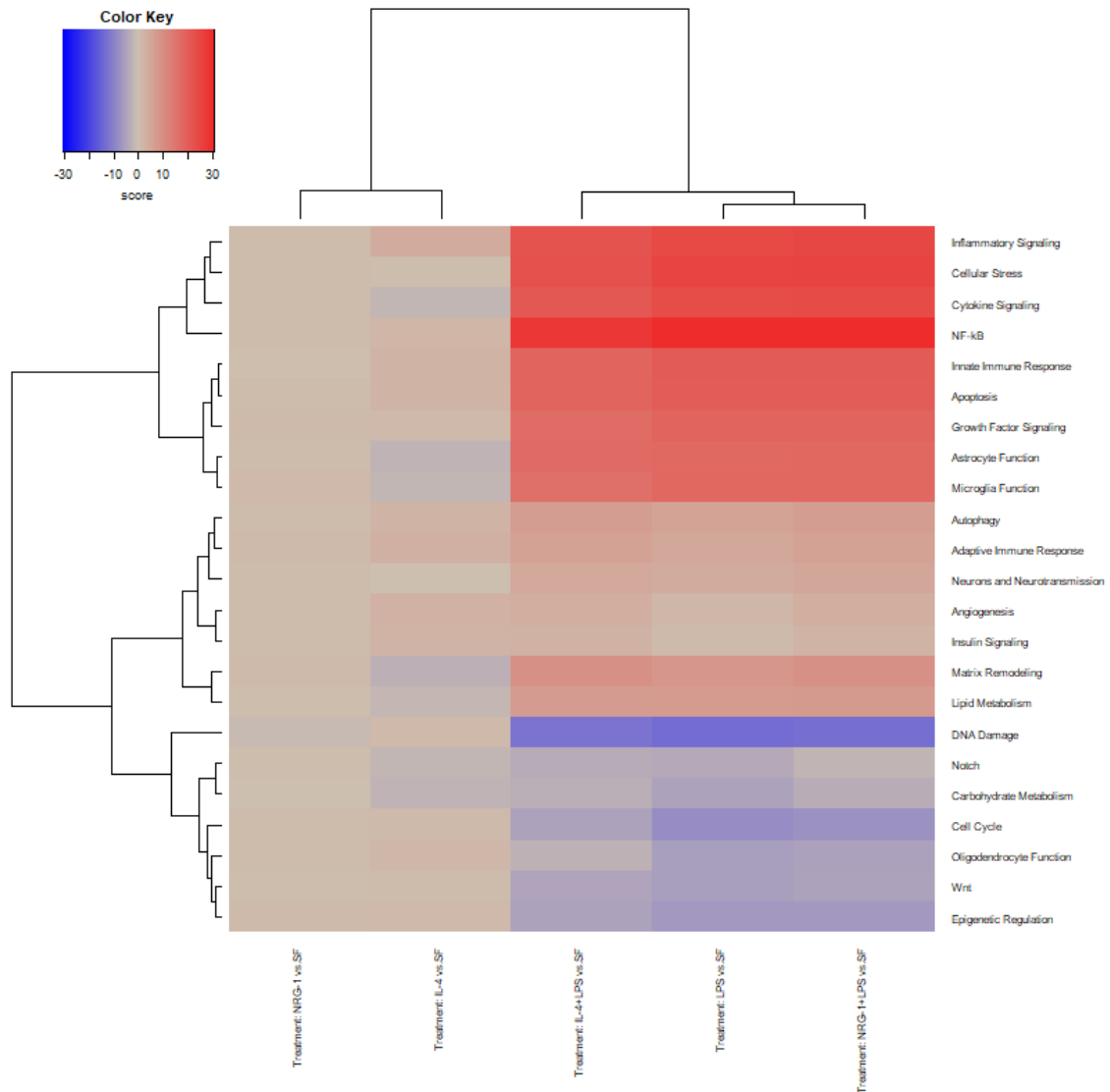
Figure 4-4 Hierarchical clustered dendrogram of directed significance scores.

Dendrogram is a visual representation of directed significance score. All scores shown have unstimulated (SF) condition as the baseline. Clustering places most similar conditions next to each other in the vertical direction and most similar gene sets together in the horizontal direction. Red = downregulated Blue = upregulated. Color intensity indicates strength of regulation.

LPS induces the greatest change in gene expression, so in highly dimension-reduced data such as significance scores or PCA the smaller changes due to co-treatment may be masked because these changes are fewer and smaller

in magnitude. To look more specifically at how NRG-1 changes LPS-induced

gene expression, we looked only at the LPS datasets (LPS. IL-4+LPS, NRG-

1+LPS) and recalculated significance of our gene sets setting LPS as the

baseline (Table 4-19

Pathway Scores Generated by Advanced Analysis.

Table 4-19
Pathway Scores Generated by Advanced Analysis

Pathways	Upregulated	Downregulated	ΔLPS	ΔIL-4+LPS
Adaptive Immune Response	x		0.545	0.116
Angiogenesis	x		0.516	0.13
Astrocyte Function	x		0.239	0.77
Autophagy	x		0.701	-0.103
Carbohydrate Metabolism		x	-0.996	1.034
Matrix Remodeling	x		1.271	0.008
Notch		x	-1.149	-0.357
Oligodendrocyte Function		x	-0.552	2.287
Wnt		x	-0.352	0.542

The most highly involved gene sets for NRG-1 treatment are angiogenesis,

microglia function, NF-kB, matrix remodeling, and growth factor signaling. IL-

4 treatment resulted in equal or higher degrees of pathway involvement than

NRG-1 for every pathway except angiogenesis, but there is overlap in most

involved pathway for each treatment, including microglia function, matrix

remodeling, inflammatory signaling and NF-kB. However, many pathways

highly altered by IL-4 treatment rank among the least involved for NRG-1

treatment, including cellular stress, oligodendrocyte function, and astrocyte

function, indicating NRG-1 treatment does not have a strong effect on these key anti-inflammatory functions. The greatest difference show up in IL-4's most highly involved pathways, indicating that NRG-1 treatment largely does not induce gene expression in the most highly regulated anti-inflammatory pathways.

The directed significance scores show that NRG-1 weakly represses one pathway while IL-4 represses 10, with seven of those being more strongly repressed. The strongest repressions are metabolic pathways, astrocyte function, and inflammatory signaling and strongest activation is oligodendrocyte function. Taken together, it is clear that NRG-1 does not engage in large-scale pathway downregulation characteristic of anti-inflammatory gene expression, but the small-scale changes that it does activate are in pathways that provide trophic support such as angiogenesis, and matrix remodeling.

PATHWAY ANALYSIS

We can look at a pre-determined set of genes and evaluate the tendency of genes within this set to be differentially expressed with regards to our baseline. The global significance score of a gene set is a measure of how much the gene set is differentially expressed for a given treatment but does not give the direction of the gene expression change. Therefore, we will refer to global significance score as a measure of gene set involvement without indicating up regulation or downregulation. Incorporating up regulation and

downregulation relative to the baseline condition can be looked at with the directed significance score. Downregulated genes have a sign in the negative direction while upregulated genes are positive. A pathway shows strong repression or activation when the directed score is similar in magnitude to its global score.

Global significance score is calculated as the root-squared mean of the summed t-statistics of all genes within the gene set.

The pathways with the most differential change in gene expression will rank highly in global significance. These pathways are important because they indicate a gene set is altered by our treatment. If the global score is high but the pathway is both activated and repressed, the directed significance score will be lower than the global score. A gene set will be considered up or downregulated when the global and directed scores are similar.

Genes were cross-referenced in the KEGG database and pathways with 80% or greater coverage within our datasets were chosen for further analysis. KEGG pathways are drawn by humans and encoded with literature-based evidence. Overlaying gene expression data onto KEGG pathways allows exploration of connectivity relationships. Nothing how gene expression changes within our dataset activate or repress branches of pathways allows us to propose 2 key additional pieces of information

    1. We can identify important nodes within the pathway

2. We can interpret more exactly how gene expression changes result in functional outcomes

Pathways are visualized through the pathview function in R. and colored based upon the change in gene expression relative to baseline. Significance was defined as a p-value of less than 0.05. Only significant genes are colored.

Flt1, also known as VGFR1, a receptor for adhesive molecules is downregulated according to the neuroinflammation panel from NanoString, and minimally increased according to inflammation NanoString. IL-4 has a strong inducing effect. Expression of this receptor plays a role in microglial chemotaxis and has been shown to be pathological in Alzheimer's.
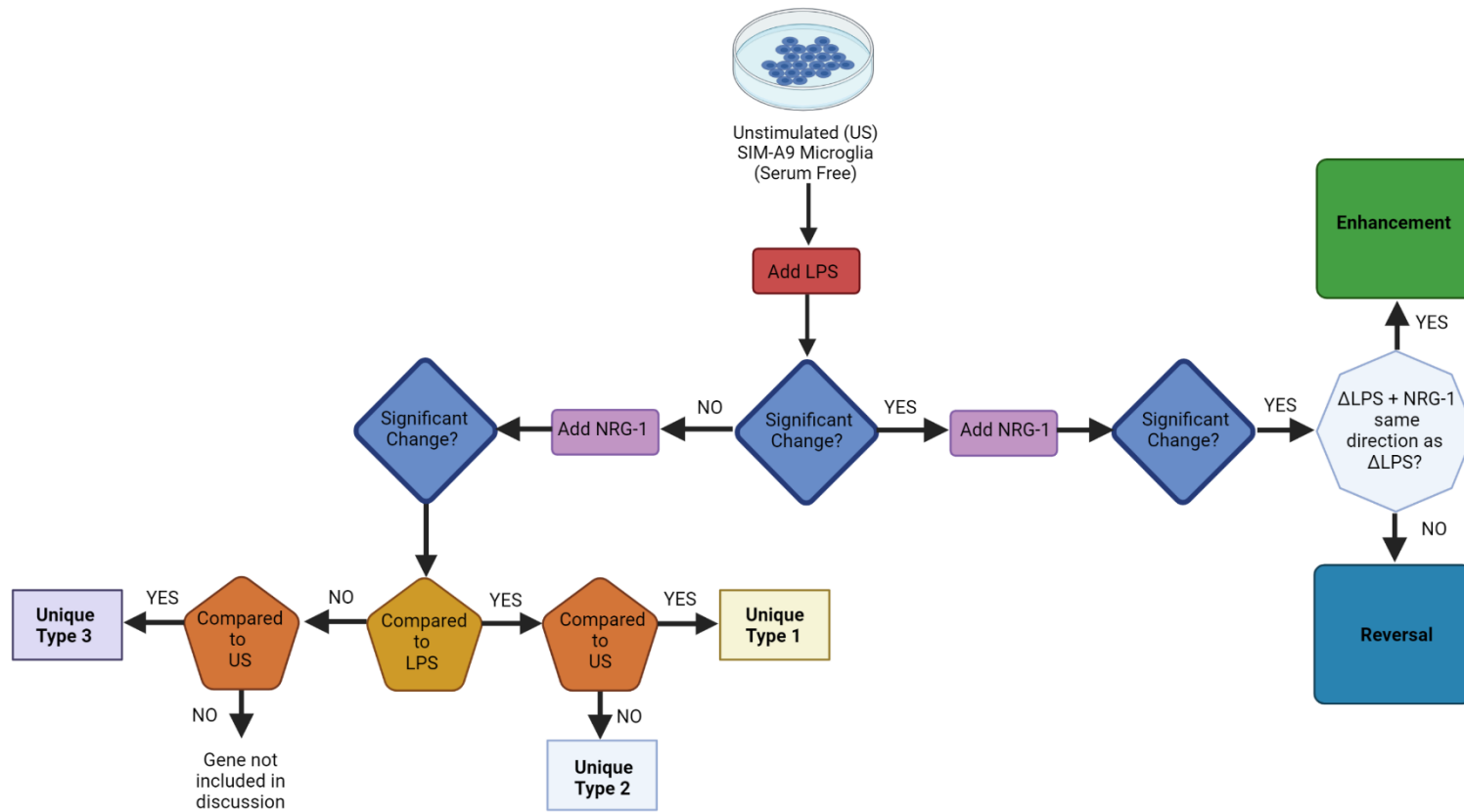
Figure 3-3 Flow chart outlining gene set categorization.

Over-Representation Analysis of GO terms and Pathways with PANTHER

Using the analytical framework developed in Chapter 3 (referred to in Figure 3-3, reproduced above), further analysis was performed using PANTHER (described in PANTHER Methods) to determine over-represented GO terms and pathways for each type of gene regulation.

<u>LPS-Induced Gene Expression Revered by NRG-1</u>

We compared the lists of non-overlapping genes that reversed LPS-induced changes in LPS + NRG-1 and LPS + IL-4 conditions, to see what kinds of genes were being reversed by each stimulus. The top GO terms where NRG-1 was significant and IL-4 was not significant include DNA integrity checkpoint and cell cycle checkpoint. For apoptotic signaling, both were significant.

There were no significantly enriched pathways in either the reactome or the PANTHER pathways databases for the list of NRG-1 reversed genes, indicating that there is not one system in which these genes are working. In contrast, genes reversed by IL-4 stimulation were enriched within three Panther pathways and several reactome pathways. The panther pathways were the FAS signaling pathway, p53 pathway, and Inflammation mediated by chemokine and cytokine signaling pathway. Reactome pathway reactions that were enriched for IL-4 reversed genes included those for IL-1 signaling, platelet activation, DNA repair and SUMOylation. These pathways are

involved in inflammatory cytokine cascades and subsequent signaling. That IL-4 plays a role in reversing inflammatory signaling corroborates the current IL-4 anti-inflammatory mechanism. However, NRG-1 does not play a significant role in these highly over-represented anti-inflammatory mechanisms.

LPS-Induced Gene Expression Enhanced by NRG-1

The influence of NRG-1 on LPS-induced gene expression was overwhelmingly towards enhancing the changes as opposed to reversing them. No pathways were significantly involved with genes enhanced by IL-4. NRG-1 enhanced genes were significantly over-represented in the apoptosis pathways, suggesting a role for NRG-1 in altering cellular reactions to apoptotic signaling.

Reactome pathways indicate more specific branches of the pathways that may be altered. Gene changes enhanced by NRG-1 were overrepresented in regulation of TNFR1 signaling while no IL-4 gene enhancements were a part of this pathway. In addition, only NRG-1 and not IL-4 enhanced gene changes within the Death receptor signaling. Both stimuli resulted in enhancements of the MyD88-independent and TRIF – mediated branches of TLR signaling. TRAF6-mediated induction of NF-kB and MAP kinases was also enriched. These results suggest that NRG-1 can preferentially push signaling events along particular branches within NF-kB, MAPK, and other transmembrane signaling.

Gene Expression Uniquely Altered by NRG-1

The third classification of stimulus-induced alteration of gene expression is the genes which are not significantly changed by LPS but did show significantly different levels of expression for the combinatorial treatment. Pathway analysis with PANTHER pathways determined that the TLR signaling pathways and apoptosis pathways were over-represented within genes uniquely regulated by NRG-1+ LPS while no pathways were significantly overrepresented for IL-4 + LPS.

Analysis in reactome showed that enriched pathway branches for NRG-1 uniquely regulated genes were inhibition of Caspase 8 via Ripk1 within necrosis signaling, positive regulation of JNK signaling via phosphorylation by activated TAK1, and TNFR1 signaling within the NF-κB pathway. Similarly, no GO terms were enriched for unique IL-4 + LPS genes. On the other hand NRG-1 top GO terms included are listed in Table 4-20.

Table 4-20 Top GO Terms for Genes Uniquely Regulated by NRG-1

NIK/NF-kappaB signaling
positive regulation of I-kappaB kinase/NF-kappaB signaling
tumor necrosis factor-mediated signaling pathway
positive regulation of interleukin-8 production
JNK cascade
positive regulation of JUN kinase activity
positive regulation of NF-kappaB transcription factor activity

<u>Total Differences Influenced by NRG-1</u>

Breaking gene sets into reverse, enhanced, and unique contributions gives some representation of how stimulus is altering LPS-induced changes. However, it is also useful to look at the total change, because more than one type of regulation can be represented in a single system or pathway. To get a clearer picture of the most involved pathways following combination stimuli, we perform the same over-representation analysis, but using a list of all non-overlapping changes in gene expression.

Fas receptor (CD95, tumor necrosis factor receptor superfamily member 6) is a death receptor (DR) expressed on cell surface, and activation triggers a signal transduction pathway leading to apoptosis.

NF-κB (nuclear factor-kappa B) is a rapidly acting primary transcription factor found in all cell types. It is involved in the cellular responses to stimuli such as cytokines and stress and plays a key role in regulating the immunological response to infections.

NRG-1 Effects on NF-kB Pathway

The NF-kB pathway is involved in cell survival and expression of pro-inflammatory mediators. NF-kB signaling can be activated via many different transmembrane stimuli, and it is likely that different stimuli result in different generally this pathway consists of two main routes, the canonical pro-inflammatory pathway. In Figure 4-5 KEGG pathway for NF-kB colored to demonstrate gene expression changes induced by NRG-1 treatmentwe see

an increase in expression of pro-survival genes and activators of the non-

canonical pathway involving p100/RelB. This complex is downstream of NIK,

which is known to interact directly with NRG-1 initiated ErbB4 signaling

cascade.

Work in the cancer field has published reports that NRG-1 signaling through

ErbB receptors can activate NF-kB-inducing kinase (NIK), suggesting the

involvement of non-canonical NF-kB signaling. Previous published work in

our lab using N9 cells established that NRG-1 treatment increased nuclear

translocation of the NF-kB p52 subunit, indicative of non-canonical NF-kB

activation(Simmons, Surles-Zeigler et al. 2016). Our finding here

corroborates well with previous work, as we see an increase in p52 gene

expression. In addition, we also see an increase in factors upstream of p52

within the non-canonical branch of NF-kB signaling pathway including p100

and IKKα, further suggesting that NRG-1 is working through this non-

canonical mechanism.

Traf6 is a part of transmembrane signaling complexes downstream of many

different receptor types and is a major pre-cursor to canonical NF-kB

signalling. Suppression of LPS-induced Traf6 expression and ubiquitination

has been shown to attenuate Mapk and Akt pathways and lead to regulation

of inflammation(Jakus, Kalman et al. 2013). Traf6 is the only LPS-Induced

gene downregulated by NRG-1 This regulation may indicate that NRG-1

reduces microglial sensitivity to receptor signaling downstream of pro-

inflammatory factors such as IL-1β. Such a mechanism would shift NF-kB away from the canonical transcription branch and also close pro-inflammatory positive feedback loop in which canonical transcription generates pro-inflammatory cytokines, which in turn activate additional canonical NF-kB activation. The overall effect of this Traf6 downregulation would likely be anti-inflammatory via reduction in proinflammatory signaling.
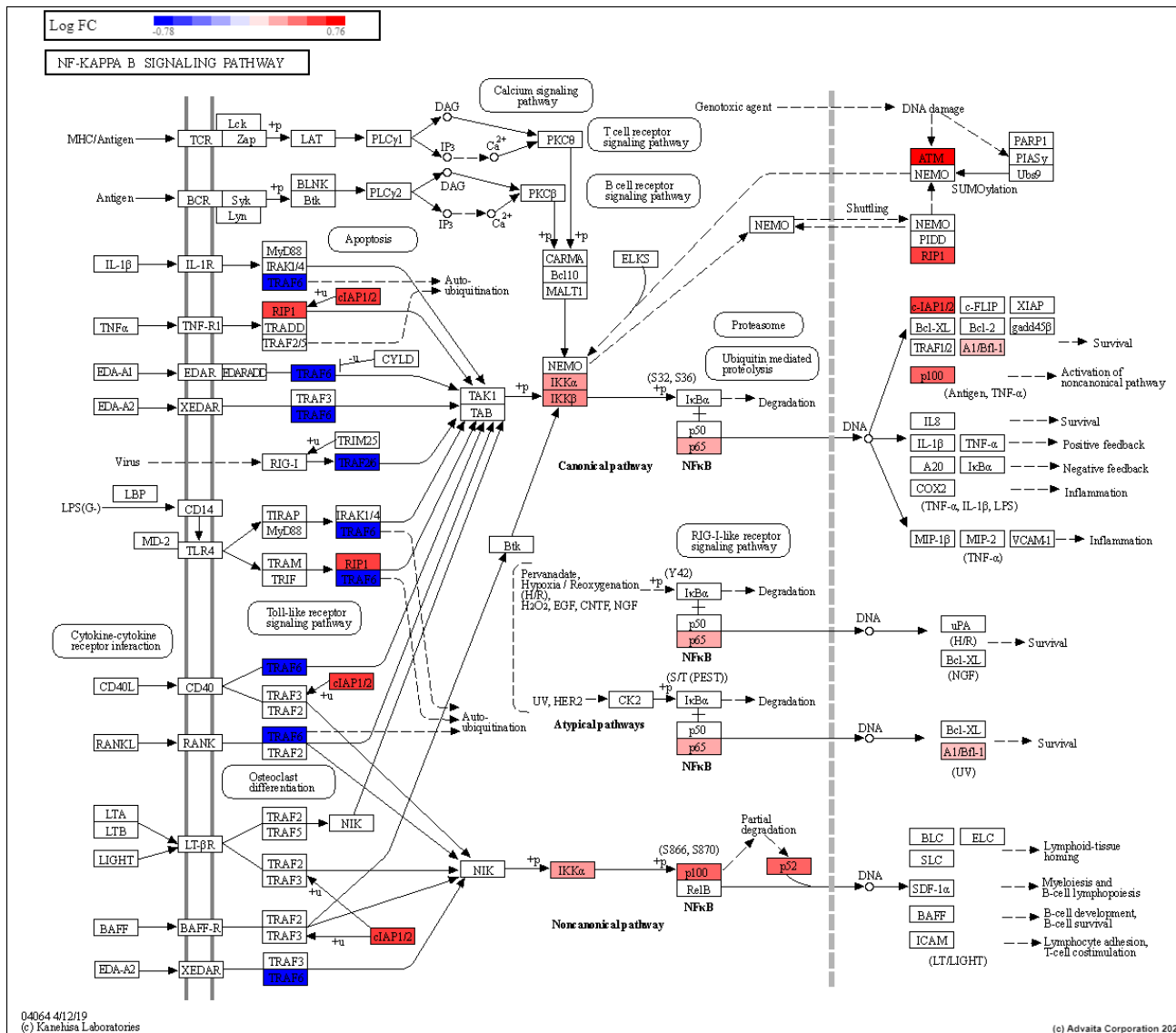
Figure 4-5 KEGG pathway for NF-kB colored to demonstrate gene expression changes induced by NRG-1 treatment

This pathway map shows connectivity of the NF-kB network. Colorized by fold change in NRG-1 + LPS condition compared to baseline of LPS alone. Pathway supplied by KEGG.. Red = upregulated by combined NRG-1 treatment Blue = downregulated by combined NRG-1 treatment.

CONCLUSION

In this chapter we expanded the genes included in our analysis from traditional markers of inflammation (discussed in Chapter 3) to include genes that are important within neuropathology and metabolism. In the previous chapter we concluded that the influence of NRG-1 on gene expression included a mix of typical anti-inflammatory changes, in line with the influence of IL-4, and typical pro-inflammatory changes, like those seen following LPS exposure. In this chapter, analysis of 757 gene changes within the expanded NanoString Neuropathology Panel confirmed that the influence of NRG-1 included a subset of IL-4-like changes, LPS-like changes, and changes that were unlike either of these canonical inflammatory state inducers. To evaluate the contributions of each of these gene-level changes to the overall inflammatory state of the microglial cell, we performed over-expression and pathways analyses. We found that NRG-1 enhances specific aspects of the LPS-induced response, altering gene expression within the pathways for NF-κB and apoptosis, resulting in the upregulation of genes that are imperative for immune cell survival.

Our analysis of NRG-1 induced gene regulation compared NRG-1 and LPS stimuli together to LPS stimulus individually allowed us to categorize NRG-1 changes into those that reversed LPS-induced changes, enhanced LPS-induced changes, or induced novel NRG-1-specific changes within genes that were not altered by LPS. Using this framework, we identified that NRG-1

results in almost twice as many enhancements to LPS-induced gene expression changes as compared to the number of genes enhanced by canonical anti-inflammatory IL-4. In contrast to overwhelming majority of IL-4's influence was a reversal of LPS-induced expression changes. The most overrepresented GO terms within set of genes uniquely regulated by NRG-1 were for positive regulation of I-κB kinase/NF-κB signaling, apoptosis and IL-8 production. These observations suggest that NRG-1 appears to enhance a portion of the microglial cell's natural response to pro-inflammatory stimuli as opposed to reversing or tamping down the pro-inflammatory response. In conjunction, NRG-1 activated unique signaling within the NF-kB pathways and regulation within the process of apoptosis.

NRG-1 exerted identifiable effects on the NF-kB system. NF-kB influences microglial inflammatory states by tweaking cell sensitivity to apoptotic signaling and increasing likelihood of survival pathways.

CHAPTER 5 CONCLUSIONS AND FUTURE WORK

The most compelling results from this work are that NRG-1 enhances NF-kB and apoptosis pathways that are triggered by pro-inflammatory stimulus and are critical to cell survival. Specifically, NRG-1 pushes NF-kB signaling towards the non-canonical arm which results in increased expression of genes that encourage cell survival. Finally, one potential marker of NRG-1 stimulation which was significantly upregulated in both unstimulated cells and when NRG-1 was delivered in conjunction with pro-inflammatory signals was CD33. This molecule mediates cell-cell interactions and plays a role in maintaining the resting state of immune cells. NRG-1 stimulated increases in this cell surface protein may indicate how NRG-1 achieves resolution of inflammation and reduced inflammatory gene expression *in vivo.*

I propose that NRG-1 does not significantly alter pro-inflammatory responses, but instead achieves reduced levels of inflammation *in vivo* by encouraging resolution of inflammation. This is achieved through several factors working in tandem. Internally NRG-1 tweaks microglial sensitivity to cell death pathways by decreasing apoptosis and increasing signaling leading to immune cell survival.

Why increased immune cell survival results in ani-inflammatory outcomes is up for speculation, but I suggest that it may have to do with allowing cells to engage autophagy.

This work was developed off previous studies in our lab in which NRG-1 was used as a treatment for stroke. In the *in vitro* work presented here, we model chemically induced benchmarks of pro-inflammatory and anti-inflammatory states. However, stroke pathology includes additional components such as hypoxia, which likely contribute additional unique features and inflammatory stimuli which were not taken into account within the model used here. Future work would include total transcriptomic analysis and proteomic analysis, as well as functional assays. Proteomics is much more able to identify autophagy, and therefore could investigate a link between immune cell survival and autophagy and anti-inflammatory effects. Finally, functional assays could assess if NRG-1 treatment really does enhance immune cell survival.

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