DISSERTATION

INFLAMMATORY MODULATION OF SYNOVIAL FIBROBLASTS IN RHEUMATOID ARTHRITIS

Submitted by

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ABSTRACT

INFLAMMATORY MODULATION OF SYNOVIAL FIBROBLATS IN RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) has a prevalence of 1-2% and is one of the more common causes of chronic morbidity among people over 65 years of age. It is characterized by hyperplasia of fibroblast-like synoviocytes (FLS) within the synovium and recruitment of multiple leukocyte populations that drive the inflammatory process. Although disease etiology is unknown, it is thought that both genetic and environmental factors trigger the onset of RA. A key mediator of cellular inflammation and joint destruction in rheumatoid arthritis (RA) is the presence of fibroblast-like synoviocytes (FLS), a unique cell type that distinguishes RA from other inflammatory conditions of the joint. Due to their presence within the disease, FLS represent a possible target for next generation RA therapeutics, used in conjunction with immunomodulators, to control disease pathology without augmenting immunosuppression.

The complex network of signal transduction pathways controlling FLS include inflammatory proteins such as cytokines, matrix metalloproteinases, cyclooxygenase (COX-2), mitogen-activated protein kinase (MAPK), nuclear factor kappa-B (NF-kB), and janus kinase (JAK-STAT), all of which have been implicated in the pathogenesis of RA. Under rheumatoid conditions, FLS express the tumor necrosis factor (TNF)-recognition complex (TNFR1, TNFR2, VCAM-1 and ICAM-1), which induces local macrophage activation and leads to downstream NF-kB signaling, that is partially responsible for propagating inflammatory damage within the joint. It is postulated that specific inhibition of NF-kB signaling in this system could mitigate FLS-driven inflammation without the negative off-target effects of global immune suppression.

We investigated into C-DIM mechanism of action, DIM-C-pPhCl was examined in the RAW264.7 macrophage cell line treated with LPS to stimulate cytokine production. DIM-C-pPhCl treatment reduced the expression of inflammatory proteins such as NF-κB, iNOS, COX-2, and prostaglandin production following LPS stimulation. In addition, DIM-C-pPhCl treatment increased the amount of nuclear p65 and Nurr1 protein. As a final investigation into the mechanism of action of C-DIM12, was examined in primary murine synovial fibroblasts treated with TNF-α to stimulate adhesion molecules and cytokine productions. These experiments provide evidence that NF-κB directly mediates the induction of VCAM-1 in synovial fibroblasts by TNF-α and furthermore C-DIM12 suppresses this activation. Demonstrating that C-DIM12 induces activity in synovial fibroblasts through a Nurr1 dependent mechanism. Providing a novel mechanism to decrease expression of NF-κB regulated inflammatory genes in synovial fibroblast cells relevant to degenerative joint diseases.

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DEDICATION

I dedicate this dissertation in loving memory of my grandmother, Madar Sima, who was a a parent, a teacher and a friend.

You taught me to hold my head up high and love my life, family and forever show gratitude for all that is going well and to live each day making it count and mean something. It was a gift to have you in our lives and watch you preserve under all seasons of change, you are a true symbol of strength. You always believed in me, even when I didn't believe in myself. You were there when I couldn't relate to anyone or anything in my life.

Your amazing grace, style and humor showed me all I wanted to become, there is not a day that goes by that I don't smile with gratitude from the light you brought into my life. My heart breaks with thoughts of missing you, but I have faith that you are in a better place, a place of love, fills my heart with comfort. I hope one day I can return the love you showed me and be a grandmother who changes lives. There is no greater gift on this earth than to be loved, but to be loved by a grandmother is something to behold.

Madar Sima, you are my treasure and I will always carry you close in my heart. Every day. Every step. Forever and ever. I love you always

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LITERATURE REVIEW

1. INFLAMMATION

1.1 IMMUNE MEDIATION IN THE PERIPHERAL SYSTEM

Inflammation is an essential immune response enabling survival in response to infection, injury and /or stress. It also helps to maintain tissue homeostasis under a wide variety of noxious conditions. Although inflammation is a protective response, it can come at the cost of a transient decline in tissue functions and cellular homeostasis (Medzhitov, 2010). When an acute inflammatory response is triggered, it typically lasts for a short period and is regulated by a negative feedback signals. Chronic inflammation involves dysregulation of this feedback mechanism, which is believed to be a key pathophysiological mechanism in various health disorders (Coussens and Werb, 2002). As resident immune cells, macrophages are ubiquitously-distributed and play important roles in inflammation. Functions of these cells include antigen presentation, phagocytosis and secretion of different types of cytokines and chemokines to regulate inflammation. In metastatic tumors, they elicit a pro-inflammatory microenvironment for cancer cell growth (Essers et al., 2013). In response to tissue injury, a multifactorial network of chemical signals initiate and maintain a host response designed to heal the afflicted tissue. These chemical signals result in activation and directed migration of leukocytes (neutrophils, monocytes and eosinophils) from the venous system to sites of damage. Activated tissue mast cells also contribute to the host injury response. For neutrophils, a four-step mechanism is believed to coordinate recruitment of these inflammatory cells to sites of tissue injury and to the provisional extracellular matrix (ECM) that forms the scaffolding on which endothelial and

fibroblast cells proliferate and migrate. This provides an origin for reconstitution of the normal microenvironment (Talmor, M. et al., 1998). The steps involved include: activation of members of the selectin family of molecules that facilitate adhesion to the vascular endothelium; triggering of signals that activate and upregulate leukocyte integrins mediated by cytokines and leukocyte activating molecules; and immobilization of neutrophils on the surface of the vascular endothelium. The latter is accomplished with tight adhesions formed through integrin binding endothelial vascular adhesion molecule -1 (VCAM-1). Chemokines recruit downstream effector cells and dictate the evolution of the inflammatory response (Rossi, D. and Zlotnik, A., 2000). Therefore, profiling persistent cytokines and chemokines at an inflammatory site is important in the development and understanding of chronic disease.

1.2 MACROPHAGE CELLS

Macrophages are a diverse set of cells that constantly shift their functional state in response to changes in tissue physiology or environmental challenges (Coussens, LM. and Werb Z., 2002). They differ morphologically and phenotypically in organs such as brain, lung, liver, spleen and gut and interact with matrix and other cell types. They are active in biosynthesis and express a wide range of receptors. Macrophages recognize foreign and as well as normal and abnormal cells and host-derived products. They react through constitutive and induced endocytosis, phagocytosis, and secretion of various products including cytokines, growth factors and metabolites. As the first responder of injury and infection, macrophages perform trophic and homeostatic roles in removal of apoptotic cells (Jackaman et al., 2017). They contribute to tissue remodeling, providing a host defense within innate and adaptive immunity as well as defending against many different disease processes (Steinman and Idoyaga, 2010).

Macrophages are commonly grouped into two broad subsets: "classically activated" M1 and "alternatively activated" M2 macrophages (Mantovai et al., 2004). Macrophages are activated through stimulation with interferon gamma (IFN-γ) alone or combined with TLR ligands such as lipopolysaccharide (LPS) or PAMPs, which increase STAT1 and IRF5 transcription factor activity (Martinez, F.O. and Gordon, S., 2014). Both M1 and M2 macrophages contribute to the production of pro-inflammatory cytokines and provide cytotoxic defenses against bacteria, protozoa, viruses and anti-tumor immunity (Mantovai et al., 2004). M1 macrophage activation is mainly achieved by IFN-γ and LPS; their main function is to secret reactive oxygen and nitrogen intermediates in addition pro-inflammatory cytokines (IL-6, IL-8, IL-1β, IFN-γ and TNF-α), and chemokines (CXCL9, CXCL10, CXCL11, CXCL16, CCL2, CCL3 and CCL5) (Mantovani et al., 2004; Martinez, F.O. and Gordon, S., 2014). Unlike M1 macrophages, M2 macrophages show an anti-inflammatory phenotype and are found in parasitic infection, allergy, tissue remodeling, tumor development and waste elimination processes following acute phase inflammation (Martinez, F.O. and Gordon, S., 2014). Known as the tissue resident macrophages, M2- like macrophages are pivotal in homeostasis maintenance. *In vitro*, they can be subdivided further based on their response to stimuli; stimulation can lead to activation of IRF4, STAT3, STAT6, and PPARγ transcription factors (Mantovani et al., 2004; Satoh et al., 2010). The plasticity of macrophages makes it difficult to assign specific biochemical markers to each population; however, the potential benefits in understanding macrophage heterogeneity is enormous because these cells can be biomarkers of diseases and can be used as surrogate markers of protection following drug treatment or even vaccination.

2. INFLAMMATION IN RHEUMATOID ARTHRITIS

2.1 PATHOPHYSIOLOGY OF RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a best identified as an autoimmune disease characterized by synovial inflammation and hyperplasia, autoantibody production, cartilage and bone destruction and multiple systemic features, including cardiovascular, pulmonary, and skeletal disorders. The development of RA involves a complex interaction of genotype, environmental triggers and chance. Association with the human leukocyte antigen (HLA)-DRB1 locus has been confirmed in patients who are positive for rheumatoid factor or ACPA. Moreover, alleles containing a common amino acid motif (QKRAA0 in the HLA-DRB1 region) have been confirmed as indicators of susceptibility to development of RA (MacGregeor et al., 2000). Infectious agents (e.g. Epstein-Barr Virus, cytomegalovirus, proteus species, and *Escherichia coli*) and their products (e.g. heat-shock proteins) have also been linked with RA. While the underlying mechanisms of this effect remain unknown, some form of molecular imitation is postulated (Kamphuis et al., 2005). While these may provide susceptibility to RA, the etiology of RA is still largely unknown. Generation of an immune response within the host tissue appears to be common to all RA cases.

2.2 INFLAMMATORY SIGNALING IN SYNOVIAL FIBROBLASTS

Although the etiology of RA is unknown, the role of synovial fibroblasts as the resident cells of the synovial tissue produce a large array of matrix proteins (e.g. collagens and fibronectin), contributing to the maintenance of overall joint integrity. A key mediator of cellular inflammation and joint destruction in rheumatoid arthritis (RA) is the presence of fibroblast-like synoviocytes (FLS), a unique cell type that distinguishes RA from other inflammatory conditions of the joint.

They function as innate immune cells in that they attract neutrophils and respond to Toll-like receptor stimulation with production of pro-inflammatory cytokines (Hardy et al., 2013). Pro-inflammatory mediators tumor necrosis factor alpha (TNF-α) and interleukin (IL-1) are released by macrophage cells to promote synoviocyte cell proliferation. This results in the release of matrix-degrading enzymes and the induction of pro-angiogenic factors and secretion of cytokines and chemokines (Aherne et al., 2009). These pro-inflammatory cytokines are master regulators of chronic inflammation and tissue destruction in RA. In response to these cytokines, FLS produce chemokine, and adhesion molecules that further promote inflammation, hyperplasia, and cartilage destruction (Choy and Panayi, 2001; Iwamoto et al., 2008; Jones et al., 2013). Several lines of evidence have indicated that synovial fibroblasts can also become activated under disease conditions and can contribute directly to inflammatory damage within the joint.

3. MOLECULAR SIGNALING

3.1 NF-κB SIGNALING IN RHEUMATOID ARTHRITIS

NF-κB resides in the cytoplasm in a latent form and must translocate to the nucleus to function. The cytoplasmic retention of NF-κB is achieved by interaction with inhibitory proteins known as IκB (Baldwin, AS, 1996). A wide variety of different stimuli, such as the pro-inflammatory cytokines TNF-α and IL-1, stimulate kinase protein IκB kinase (IKK). Stimulation of IKK leads to phosphorylation-targeted proteasomal degradation of IκB, allowing the active NF-κB to enter the nucleus and initiate transcription. The IKK complex core components are three different proteins including two catalytic subunits IKKα/IKK1 and IKKβ/IKK-2 and a scaffold protein NEMO/IKKγ that links the catalytic kinase subunits with the upstream activators (DiDonato et al., 1997). Experimental evidence suggests that NF-κB activation plays a pivotal role both at the stage of initiation and stage of perpetuation of chronic inflammation in RA.

3.2 NUCLEAR RECEPTORS AND INFLAMMATION

NF-κB is highly activated at sites of inflammation in diverse diseases, where it functions as a cell survival protein, involved in DNA transcription and cytokine production (Lawrence, T., 2009). NF-κB plays a crucial role in inflammation and adaptive immunity. Dysregulation is common within all inflammatory diseases, making it a desired therapeutic target. Manipulation of this pathway by overexpressing I*k*Bα or knockdown of IKK have shown promise within inflammatory models resulting in cytokine and metalloproteinase production blocks (Bondeson et al., 1999). Other studies suggest that total knockout of IKK have undesirable side effects such as vulnerability to infection and immunosuppression (Makarov et al., 2001). Recent studies have shown that members of NR4A subfamily (NR4A1/NUR77, NR4A2/NURR1 and NR4A3/NOR1) of orphan nuclear receptors are potential key regulators of cytokine and growth factor action in diseases characterized by prolonged or inappropriate inflammatory responses (Bonta et al., 2006; Holla et al., 2006; McEvoy et al., 2002; Mix et al., 2007). These sub-families are ligand-independent and constitutively active receptors; the activity of these transcription factors is controlled at the level of expression, subcellular localization and post-translational modification. The NR4A been demonstrated to be up-regulated in inflamed human synovial tissue, psoriatic skin, lung and colorectal cancer cells compared to normal tissue (McEvoy et al., 2002). These genes are rapidly activated by inflammatory mediators, suggesting their respective receptors may act as potential transcriptional mediators of cytokine signaling (Bonta et al., 2006; Holla et al., 2006; McEvoy et al., 2002; Mix et al., 2007).

The NR4A orphan nuclear receptor family has three members: Nur77 (NR4A1), Nurr1 (NR4A2) and Nor1 (NR4A3) with conserved zinc finger DNA binding domains (DBD) and a ligand binding domain (LBD; Bonta et al., 2006; Holla et al., 2006; McEvoy et al., 2002; Mix et

al., 2007). RA joint tissues produce markedly increased levels of NR4A2 compared to other NR4A family members (McEvoy et al., 2002; Mix et al., 2007). NR4A2 is encoded within an immediate early gene that is rapidly induced in cells in response to external stimuli such as cytokines. Microarray analysis has established that over-expression of NR4A2 in K4 IM synoviocytes cells induces the expression of pro-inflammatory genes, including IL-8 (Davies et al., 2005). In addition, studies in rheumatoid joint synovial tissue have shown Nurr1 to be highly expressed, and that inflammatory cytokines are capable of increasing NR4A2 expression through NF-κB and CREB dependent signaling. Therefore, the elevation in NR4A2 may lead to increased transcription of corticotropin-releasing hormone (Murphy et al., 2001), which would suppress the inflammatory response. This novel mechanism for suppression of inflammation in synovial fibroblasts poses a new possibility for therapeutic intervention aimed at reducing synovial tissue destruction in RA.

4. THERAPIES FOR RHEUMATOID ARTHRITIS

4.1 THERAPEUTICS

The main treatment goals with RA are to control inflammation and abate disease progression. Prognostic factors have been established as a clinical tool for treatment regimen in RA. Treatment includes medication, physical therapy and regular exercise. The goal is early aggressive treatment for favorable results of slowing down joint damage or stopping its progression. RA medicinal treatment can include one or more the following non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs (DMARDs), biologics and steroids.

Early research demonstrating RA progression as the result of sustained inflammation has driven the focus toward drugs that can mitigate the disease with anti-inflammatory compounds.

Studies have shown that patients with early onset of RA can use non-steroidal anti-inflammatory drugs (NSAIDs) to help alleviate arthritis pain and inflammation. To help slow disease activity, the use of corticosteroids including prednisone, prednisolone and methylprednisolone are potent and quick-acting anti-inflammatory medication. Corticosteroids are used to mitigate potentially damaging inflammation, while waiting for NSAIDS and disease-modifying anti-rheumatic drugs (DMARDs) to take effect (Bingham, C.O., 3rd., 2008).

Initial intervention of RA mitigation, physicians prescribe DMARDs such as methotrexate. DMARD agents have shown to alter the disease course and improve radiographic outcomes. The anti-inflammatory effects of DMARDs in RA appear to effect inflammatory and immune-regulatory pathways. The exact mechanism of action is unknown. By way of example, immunosuppressive and toxic effects of methotrexate have been associated with the inhibition of dihydrofolate reductase, an enzyme involved in the metabolism of folic acid. Biologic agents are engineered drugs that target specific inflammatory cells, cellular interaction or cytokines that mediate RA-related tissue damage (Deepali, S. and Brasington, R., 2012). These agents are designed to reduce the signs and symptoms of RA and slow the disease progression. The first targeted biologic for RA is Enbrel, a tumor necrosis factor (TNF)-antagonist (Smolen, J.S., et al., 2007). These drugs act by inhibiting the binding of TNF-α to its receptor. Overall these targeted biologics are highly effective in reducing RA symptoms, slowing disease progression, and improving physical functions.

4.2 FAILURE OF THERAPIES TO MEDIATE RHEUMATOID ARTHRITIS

There is still an unmet need in RA in that while most patients are able to achieve major clinical improvement, many fail to reach remission status. Unfortunately, a common pitfall of research within RA is that the etiology is multifactorial, with genetic and environmental

components leading to early immune perturbation in both the innate and adaptive immune response. Although, animal models have been used extensively in studies of RA pathogenesis, there are inherent limitations of all animal models. Rodent model have provided significant knowledge of the fundamental mechanisms underlying RA, leading to current advances in treatment. However, these models are unable to recapitulated clinical pathology. As the knowledge of etiology of RA expands, it is necessary to adapt and modify these animal models to better represent human disease. Therefore, researching RA as a systemic disease and identifying the multiple factors that are potential contributors to the disease pathology is crucial.

5. SUMMARY

Rheumatoid arthritis is one of the most prevalent autoimmune diseases in humans. It is characterized by hyperplasia of FLS within the synovium and recruitment of multiple leukocyte populations that drive the inflammatory process. Although disease etiology is unknown, both genetic and environmental factors are hypothesized to trigger the onset of RA. This onset is clinically recognized when a patient presents symptoms fulfilling disease classification criteria brought forth by the American College of Rheumatology criteria for RA. However, it is believed that disease onset may occur much earlier, even prior to symptom onset. The disease course of RA can range from mild and self-limiting to severe and progressive.

RA synovial fibroblasts represent a unique cell type that distinguishes RA from other inflammatory conditions of the joints. Macrophage cells release pro-inflammatory mediators, including TNF-α and IL-1, to promote synoviocyte cell proliferation. This process involves the release of matrix-degrading enzymes, induction of pro-angiogenic factors, and secretion of cytokines and chemokines. These proinflammatory cytokines are master regulators of chronic inflammation and tissue destruction in RA.

In response to these cytokines, FLS produce chemokines and adhesion molecules that further promote inflammation, hyperplasia, and cartilage destruction. Several lines of evidence have indicated that synovial fibroblasts can also become activated under disease conditions and can contribute to inflammatory damage at the joint. Effective treatment of RA has been hampered by the heterogeneity of the disease. When medical attention is sought, the most common diagnosis is defined as "undifferential arthritis", indicating arthritis as difficult to classify as a specific disease. Individuals with undifferential arthritis are followed over time and aren't formally diagnosed unless their symptoms persist for greater than six weeks and meet the current diagnostic criteria of RA. Once they meet the criteria, patients are administered therapies on a trial-and-error basis, with dose escalation and addition of other therapies. This ultimately leads to variation of treatments for a patient over the course of the disease.

There are several therapeutic approaches for RA, DMARDs, NSAIDs, and glucocorticoids. DMARDs target inflammation and, by definition, must reduce structural damage progression. NSAIDs reduce pain and stiffness while improving physical function; however, they do not interfere with the pathogenesis of RA and therefore are not acceptable disease modifying treatments. On the other hand, glucocorticoids offer rapid symptomatic and disease-modifying effects but are associated with serious long- term side effects.

Despite advances made over the past two decades, many questions still linger. First, we lack understanding about therapeutic targets in RA and their effectiveness at limiting the underlying pathology of the disease. Secondly, we cannot predict optimal responses or toxic risk for a given treatment; current molecular analyses have failed to answer this question.

Third, the current therapeutic standard for RA patients is to minimize disease activity, leaving many patients unable to reach this goal because they remain dependent on medication.

There is a large gap for new therapeutic development in RA, targeted to the disease pathology while ameliorating the devastating symptoms of arthritis

CHAPTER 2

METHODOLOGY: FLOW CYTOMETRY

2.1 BACKGROUND

Flow cytometry is a technique that allows for researchers to simultaneously identify, count, and characterize multiple characteristics of biological cells and other small particles. The data collected is both quantitative and qualitative. Flow cytometry allows for the simultaneous multi-parametric analysis of physical and chemical characteristics of particles at a rapid rate. The power of flow cytometry lies in the ability to analyze up to thousands of particles per second, allowing for a large data set per sample analyzed and providing a distribution of information for an entire group of cells (Wood, J.C., 1998).

Utilizing flow cytometry, an individual can measure specific properties of individual cells or particles. When a sample enters a flow cytometer, the particles are distributed within the sample line. The fluidics system aids in forming the single particle into a stream, which then can be interrogated individually by the instrument's detection system. This consists of a central core through which the sample fluid is injected and enclosed by an outer sheath fluid. Altering the sheath will determine the velocity in which the sample will flow; therefore, narrowing the sheath increases fluid velocity. Applying hydrodynamic focusing aligns the stream of particles into a single file. Once hydrodynamic focusing has been achieved, each cell/particle passes through one or more beams of focused light, or lasers, which produce a single wavelength of light at a specific frequency.

2.2 LIGHT SCATTER

Most instruments measure light scattered by the cells at right angles to the laser beam (side scatter, SS) and light scattered in a forward direction (forward scatter, FS). The amount of light scattered is affected by the size, shape and complexity of the cells or other particles being measured (Wood, J.C., 1998; Brown, M., 2000). Additionally, the amount of scatter is also dependent on the angle at which the scatter is measured; for instance, FS is sensitive to the range of angles over which the light is collected. As a result, the appearance of the FS is dependent on the instrument design and may be slightly different in different makes of cytometer. FS is most sensitive to the size of the cell while SS is most influenced by the optical homogeneity and complexity of the system (Wood, J.C., 1998; Brown, M., 2000).

2.3 FLUOROPHORES AND LIGHT

Fluorophores are fluorescent markers used to detect the expression of cellular molecules such as proteins or nucleic acids. The main principle of excitation and emission is the means which fluorophores can functionally accept light energy (i.e. from a laser). Emission follows excitation extremely rapidly, within nanoseconds, and is referred to as fluorescence. Before designing your protocol, it is important to understand the principles of light absorbance and emission.

Electromagnetic energy travels in waves possessing both frequency and length. These wave properties determine the color of the light (Shapiro, H.M., 1998). During excitation, fluorophore valence electrons absorb photons of light and elevate the electron energy level. During this short excitation period, some of the energy is dissipated by molecular collisions or transferred to a proximal molecule. The remaining energy is emitted as a photon to relax the electron back to the ground state. Because the emitted photon typically carries less energy, it has

a longer wavelength than the excitation photon. As a result, the emitted fluorescence can be distinguished from the excitation light. Fluorophores excitation and emission photons are cyclical, and until the fluorophores are irreversibly damaged, can be repeatedly excited. Since fluorophores can emit numerous photons through the cycle of excitation and emission, fluorescent molecules can be exploited within a broad range of research applications including flow cytometer (Wood, J.C., 1998).

Stokes shift refers to the distance between the excitation and emission wavelengths and is a key aspect in the detection of the emitted fluorescence in biological applications. Each fluorophore has a distinct Stoke shift; however, the detection of emitted fluorescence can be difficult to distinguish from the excitation light when using fluorophores with very small Stoke shifts because the excitation and emission wavelengths overlap. Conversely, fluorophores with large Stoke shifts are easy to distinguish because of the large separation between the excitation and emission wavelengths. The differences in Stoke shirts are crucial in multiplex fluorescence applications because the emission wavelength of one fluorophore may overlap, and excite another if it falls within the excitation spectrum of the neighbor molecule in the same sample but there are ways of setting controls within your assay to account for this overlap of wavelengths (Kirk, W., 2008).

2.4 GATING DATA

This is perhaps one of the most crucial concepts of flow cytometry. Before you are able to analyze your data for statistical value, you need to distinguish and quantify the different populations within your sample. In order to do this the investigator must have collected as much data as possible about the cells or particles of interest and include the right controls. This is relatively simple; the general rule of thumb within flow cytometry is referred to fluorescence

minus one (FMO; Perfetto et al., 2004). In addition, a negative control can also aid your analysis by allowing you to set negative gates and determine real populations. The first step in gating is often distinguishing the populations of cells or particles of interest within your sample; this is achieved by creating a bivariate plot displaying FS and SS properties. This will allow you to see the size and complexity within your sample and eliminate debris and any aggregates that might have occurred. Debris and dead cells often have a lower level of FS and are found at the bottom left corner of the density plots, whereas aggregates can be found at the tail end of your population of interest at the right-hand side of the density plot (Figure 2). Single parameter or univariate histograms display a single measurement parameter (relative fluorescence or light scatter) on the x-axis and the number of events (cell count) on the y-axis. The histogram expresses the data collected or a selected gated population and is a useful way to evaluate the total number of cells in a sample that possess the physical properties or expression of interest. The cells or particles that express the desired characteristic are often referred to as the positive dataset (Figure 3). Two-parameter or bivariate histograms display two measurement parameters; the parameters can be fluorescence, FCS or SSC depending on what you want to show (Figure 4). Gating is a powerful tool but in order to accurately identify the positive dataset, the experimental design needs to include appropriate controls, as discussed earlier in the chapter. This is necessary when emission spectra overlap since fluorescence from more than one fluorochrome may be detected. To account for this spectral overlap, a process known as compensation needs to occur which will ensure that the fluorescence detected in a particular detector derives from the fluorochrome that is being measures. In the example shown in Figure 5, following excitation with 488 nm light PE emission is largely detected in the detector specific for PE but the emission tail lies within the range of the bandpass filter used for detection FITC.

This will be seen as “false positive” signals in the FITC channel and fluorescence compensation is needed to correct for this overlap (Roederer, M. et al., 2001).

2.5 STATISTICAL ANALYSIS

In flow cytometry, two characteristics are generally measured for distribution, intensity and spread. The intensity of a distribution can be represented by the position of the “centre” of the distribution. The centre is usually represented mathematically by the mean, median or peak channel number; if the data is reported on a linear scale, the arithmetic mean is used; for logarithmically displayed data, the geometric mean is generally chosen. If any part of the distribution lies off scale at either end or the axis, the value for the mean channel number will be inaccurate and should not be used. The median channel can be used as long as more than half of the distribution is on scale. The peak channel number is an inaccurate measure of the centre of a distribution and isn’t a sound method of quantitation. When a Guassian (normal) distribution is reported, these three values should be equal (Overton, W.R., 1988; Wood, J.C., 1998). The spread of a distribution is usually expressed as the Standard Deviation (SD). However, in flow cytometry, the coefficient of variation (CV) is preferred because it is dimensionless and, on a linear scale therefore doesn’t depend on where in the histogram the data is recorded (Overton, W.R., 1988).

2.6 SUMMARY

Flow Cytometry is a powerful technique for analysis of multiple characteristics on single cells; it is a superior method of cell analysis but comes at a high cost. The qualitative and quantitative technique has made the transition from a standard clinical testing to a research tool. Applications of flow cytometry are utilized in Immunophenotyping, DNA and RNA contents analysis, heterogeneity of a cell population and assessment of structural and functional properties

of specific cells. Flow cytometry has the ability to discriminate thousands of cells within seconds, providing researchers with high throughput analysis and data acquisition. Despite high costs and the need for well-trained operators, flow cytometry remains the first and best choice for high-speed sample analysis.

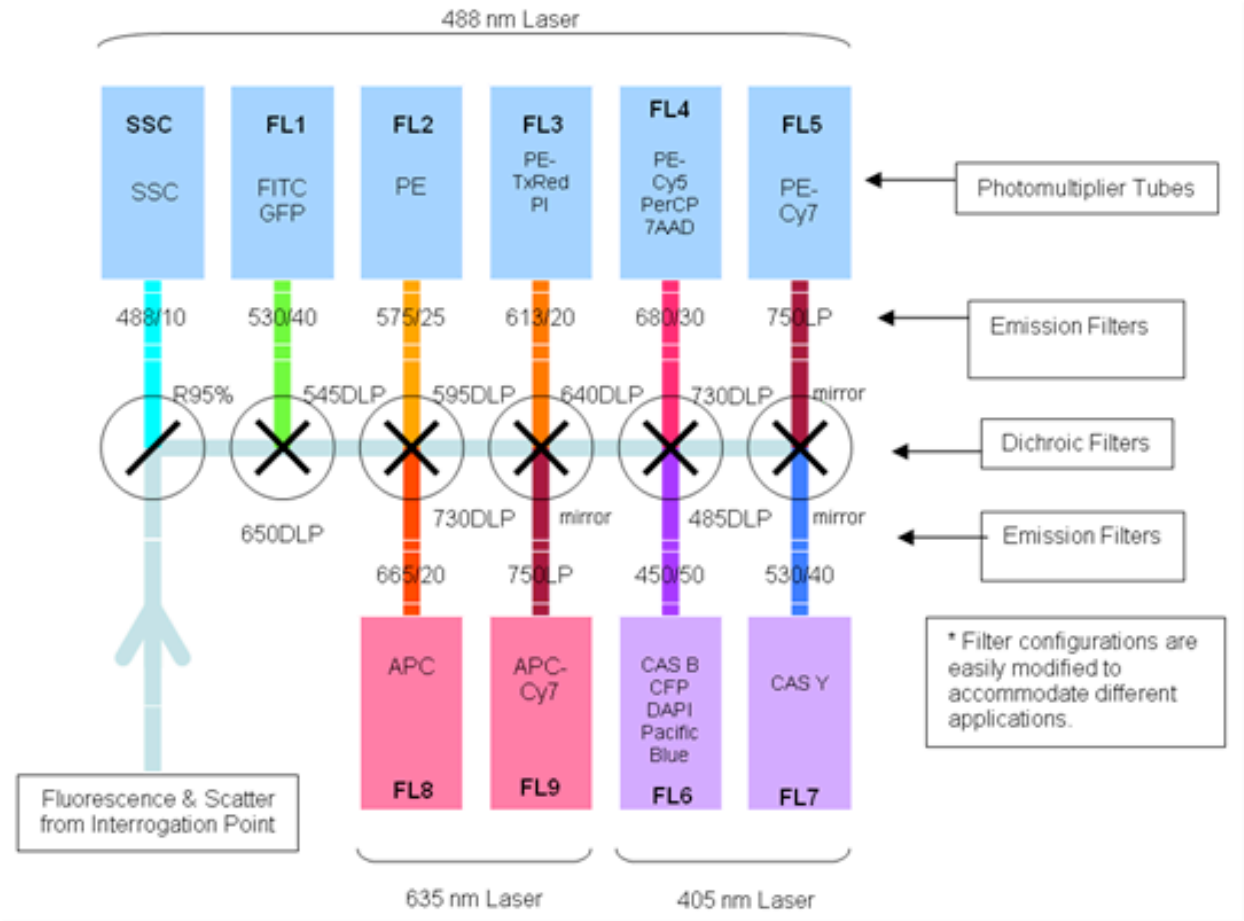


Figure 1. Schematic Overview of Typical Flow Cytometer Setup. The fluidics system pressurizes the system and transports cells/particles to the interrogation point. Lasers are used as excitation sources and their beams, along with the scatter and fluorescence, are directed within the optical system (Beckman Coulter, 2008).

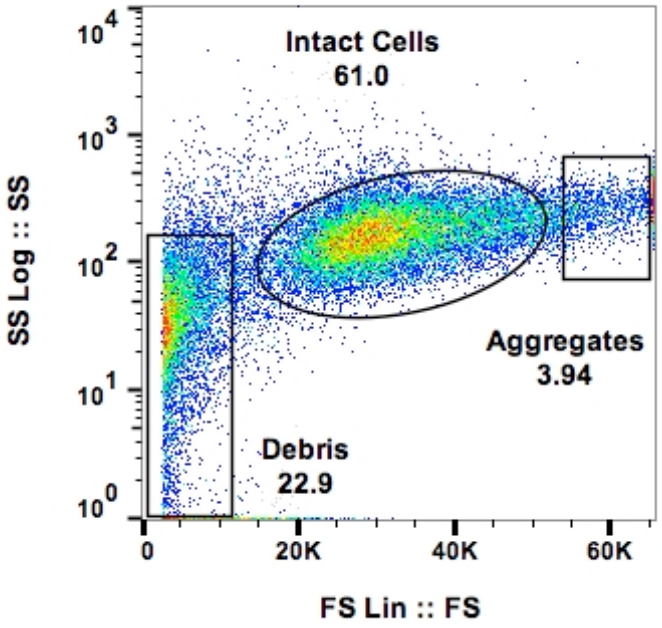


Figure 2. Gating Cell populations forward and side scatter. Bivariate plot outlining where Intact Cell population, debris and aggregates would appear on a scatter plot of forward scatter versus side scatter.

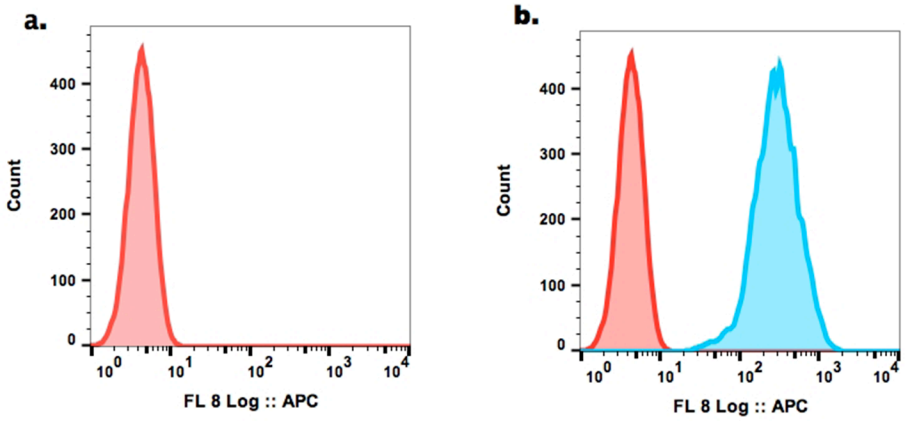


Figure 3. Single Parameter Histogram. (a) Negative control histogram (shown in red shaded area) (b) stain positive control histogram (shown in blue shaded area)

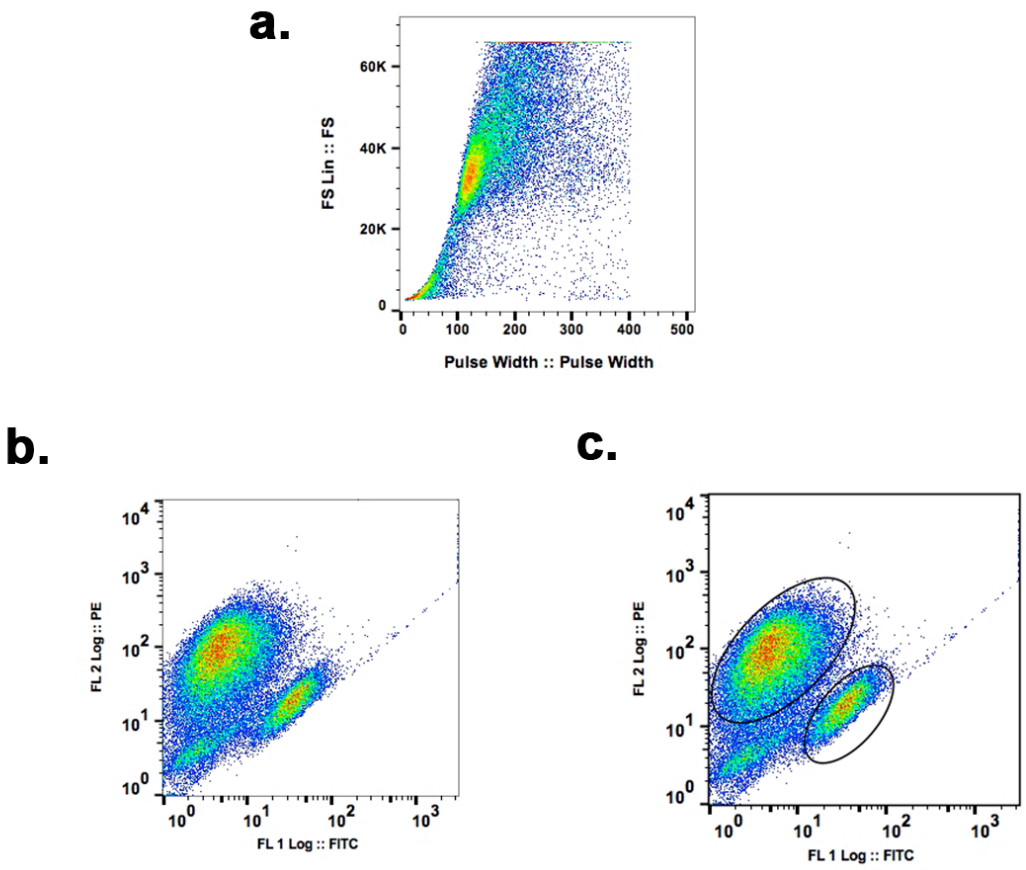


Figure 4. Two Parameter Density Plots (a) Pulse Width vs. FS discriminating doublets within a population (b) Dual stained sample FITC vs. PE (c) Dual stained gated sample of positive populations of FITC and PE.

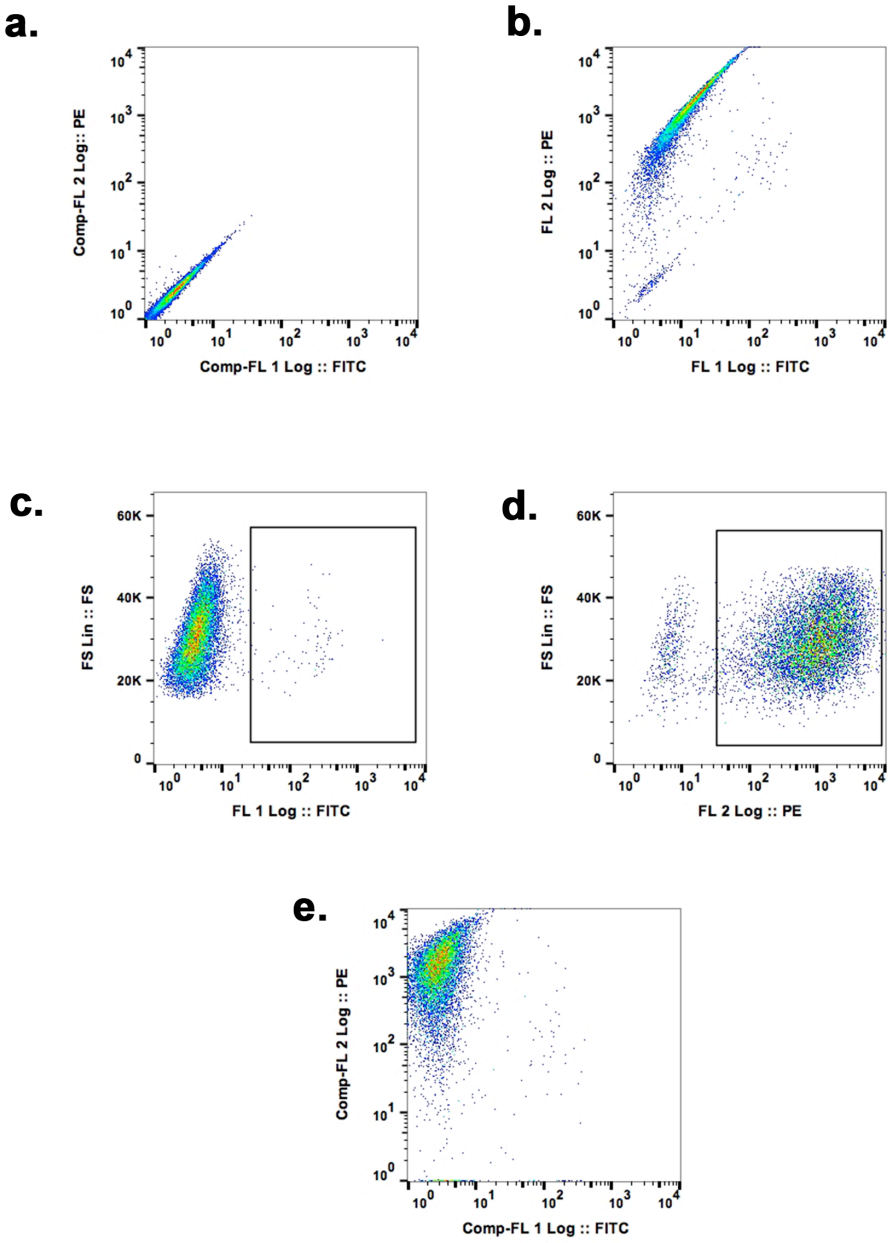


Figure 5. Compensation Bivariate Plots. (a) Negative control for PE and FITC Flour (b) uncompensated bivariate plot of samples. (c) FITC positive cells are gated (d) PE positive cells are gated (e) Compensated sample with the above controls the compensation matrix was created and took into account spectral overlap.

CHAPTER 3

1,1-BIS(3'-INDOLYL)-1-(*P*-CHLOROPHENYL) METHANE INHIBITS INFLAMMATION IN RAW264.7 CELLS BY TARGETING NF-KB, COX-2 AND INOS.

INTRODUCTION

Inflammation is an important part of the physiological defense process that is triggered by infection, tissue injury, toxin exposure, or exposure to bacterial endotoxins such as lipopolysaccharide (LPS) (Medzhitov, R., 2008 and Tabas, I., et al. 2013). However, unmitigated inflammation is implicated as the core pathology in numerous chronic illnesses such as rheumatoid arthritis (RA), cancer, diabetes, cardiovascular disease, and neurodegeneration. The key cellular players in the inflammatory process of peripheral tissue are macrophages, bone marrow-derived monocytes, that have a critical role in the immune response by producing pro-inflammatory cytokines and inflammatory mediators. Given this central role in the physiological process of inflammation, macrophages represent a strong target for therapeutic intervention to limit persistent inflammation and the resulting long-term sequelae (Sato et al., 2005; Dyke et al., 2013 and Li et al., 2016).

LPS activates macrophage cells by binding to toll-like receptor 4 (TLR4) on the surface of macrophages, which subsequently activates downstream signal transduction pathways such as mitogen-activated protein kinases (MAPKs), leading to the activation of transcriptional factors such as nuclear-factor (NF-κB) (Choi et al., 2011 and Wilson et al., 2016). LPS-activated macrophages secrete a considerable number of inflammatory mediators, such as tumor necrosis factor (TNF-α), nitric oxide (NO), interleukin-6 (IL-6), interleukin-1β (IL-1β) and prostaglandin

E_2 (PGE_2), all of which contribute to the innate response of many organisms (Shin et al., 2016 and Wu et al., 2016). Therefore, therapeutic intervention that limits these pro-inflammatory mediators may lead to successful treatments for inflammatory-derived pathologies.

The development of naturally derived anti-inflammatory compounds are of interest in an effort to prevent or minimize chronic inflammatory conditions while minimizing side effects [9,10]. One recently investigated therapeutic avenue is the use of a series of 14 novel, para-substituted diindolylmethane (C-DIM) compounds based on 3-3'-diindolymethane, an acidic condensation product of indole-3-carbinol, a phytochemical found in cruciferous vegetables (Safe et al., 2008). The C-DIM compounds, specifically C-DIM12, have shown neuroprotective efficacy in a Parkinson's disease model by decreasing inflammatory signaling in microglia, the resident macrophage-like cells in the central nervous system (De Miranda et al., 2013 and 2014). It was reported that microglia treated with C-DIM12 had decreased activation of NF-kB and its downstream signaling molecules such as inducible nitric oxide synthase (iNOS), IL-1β, and IL-6 (De Miranda et al., 2015).

Based on these results we postulated that C-DIM12 may reduce the production of pro-inflammatory signals specific to the peripheral tissue immune response such as COX-2 and prostaglandins. Using the murine macrophage cell line RAW 264.7, we assessed the ability of C-DIM12 to mitigate NF-κB, iNOS, COX-2, and prostaglandin production following LPS stimulation.

MATERIALS AND METHODS

Reagents. C-DIM12 was synthesized and characterized as described by Qin et al. (2004). LPS from *Escherichia coli 0111:b4* and all general chemical reagents were purchased from Sigma,

Aldrich (St. Louis, MO) unless stated otherwise. Antibodies for NF-κB p65, COX-2 and iNOS used in Flow Cytometry and immunofluorescences experiments were purchased from Santa Cruz Biotechnology (Dallas, TX) and BD Biosciences (San Jose, CA), respectively.

Cell Culture Experiments. Mus *musculus* macrophages (American Type Cell Culture, RAW 26.4 7, TIB-71) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 5mM sodium pyruvate. For all experiments, the cells were subjected to no more than 20 passages.

Cell viability assay. RAW264.7 cells (5×10^4 per well) were plated in 96 well plates and allowed to adhere for 18 hours. The medium was then changed to DMEM medium containing 2.5 % FBS, and either vehicle (dimethylsulfoxide [DMSO]) or various concentrations (0, 0.1, 1.0, 10 and 100 μM) of C-DIM12 in the absence or presence of 1μg/mL of LPS. After 24 hours, treatment medium was replaced with fresh medium containing 0.05mg of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolim bromide) per 100 μL and incubated for 4 hours. Medium was then removed, and 100 μL of DMSO was added to wells. The optical density of each sample was read on a plate reader at 570 nm against blank prepared from cell free wells. Cell viability was expressed as a percent of relative absorbance of untreated controls.

Inhibition of nitric oxide (NO) production. RAW264.7 cells (2.5×10^4 per well) were plated in 24 well plates and allowed to adhere for 18 hours. RAW264.7 cells were then treated with 10 μM C-DIM12 for 1 hour followed by 1μg/mL of LPS for 24 hours. The cell culture supernatants were then assayed for nitrite using the Griess Reagent Nitrite system (Cell Signaling). The

absorbance of each sample was read on a plate reader at 550 nm against blank prepared from cell free wells. NO production was expressed as a concentration (μM) of relative absorbance of untreated controls.

PGD_2, PGE_2 & PGF_2 Detection by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). RAW264.7 cells (1 x 10^6 per sample) were plated in 70 mm plates and allowed to adhere for 18 hours and then treated in absence and presence of 10 μM C-DIM12 and/or 1μg/mL of LPS. The cell culture supernatants were harvested after 24 hours, stabilized by the addition of 0.1% (v/v) butylated hydroxytoluene and stored at -80 °C. The samples were prepared for analysis by LC-MS/MS analysis using a method developed to detect PGD_2, PGE_2 & PGF_2. Samples were performed in triplicates and concentrations were expressed in pg/mL per sample.

Sample preparation was carried out by adding 2 drops of acetic acid to 2 mL of cell culture supernatant with 30 μL of 1 μg/mL of internal standard added then vortexed. Strata-C18U 500mg SPE cartridges (Phenomenex, Torrance, CA) were conditioned with 5 mL methanol followed by 5 mL of MilliQ water for each sample. The samples were then each loaded into the column and the SPE cartridge was washed with 4 mL MilliQ water followed by 5 mL of hexane. The samples were then eluted with 5 mL ethyl acetate/1% methanol. The samples were dried utilizing a nitrogen evaporator, re-suspended in 200 μL of 80/20 mobile phase (Mobile phase A: 10 mM Ammonium Acetate/0.01% formic acid in water and Mobile Phase B: methanol) and analyzed by LC-MS/MS.

Immunofluorescence Protein Expression and Translocation. RAW264.7 cells were plated on cover–glass (preconditioned with FBS) and allowed to adhere for 18 hours. Cells were then pretreated for 1 hour with 10 μM C-DIM12 followed by 1μg/mL LPS. After 6 hours the cells were rinsed with PBS buffer and then paraformaldehyde fixed for 10 minutes at 4°C. Cells were blocked using bovine serum albumin (Sigma-Aldrich) for 1 hour at room temperature followed by incubation with primary antibodies overnight at 4°C. Secondary antibody incubation was at room temperature for 2 hours with Alexa Flour 555 (Life technologies) and DAPI counterstain (Vector Laboratories, Burlingame, CA). Slides were imaged using a Zeiss Axiovert 200M inverted fluorescence microscope equipped with a Hammamatsu ORCA-ER-cooled charge-coupled device camera (Hammamatsu Photonics, Hamamatsu City, Japan) using slidebook software (version 5.5, Intelligent Imaging Innovation, Denver, CO).

FACS analysis. RAW264.7 cells (3×10^5 per well) were plated in 6 well plates and allowed to adhere for 18 hours. The cells were then pretreated with for 1 hour with 10 μM C-DIM12 followed by 1μg/mL LPS for 6 hours. The cells were then harvested and fixed with 2% paraformaldehyde for 10 minutes. Cells membranes were permeabilized with 0.01% triton X-100 in PBS for 30 minutes and stained with primary antibodies against NF-κB, COX-2 and iNOS for 1 hour at 37 °C. Secondary incubation was at 37 °C for 1 hour with Alexa Flour 647. Unstained cells were used as negative controls during acquisition. Flow cytometry was conducted on a Beckman Coulter CyAn^{ADP} flow cytometer operating with Summit v4.3 software for data collection. All further analysis was done with FlowJo (version 10.0.8; Software, Ashland, OR). Samples were run in biological triplicates with two technical duplicates.

Statistical analysis. Statistical analyses were performed using Prism (version 7.0; Graph Pad Software, San Diego, CA). Data are presented as mean ± S.E.M. Experimental group analyses were performed using a one-way analysis of variance with a Tukey post hoc test. *P < 0.05, ** P < 0.01, ***P < 0.001, **** P< 0.0001 were considered statistically significant.

RESULTS

C-DIM12 increases viability of RAW264.7 cells

The results of the MTT assay showed that C-DIM12 had no cellular toxic effects on RAW264.7 cells at concentrations up to 10 μM (Fig. 6b). At 100 μM C-DIM12, there was a decrease in cell viability (data not shown). Interestingly, C-DIM12 increased cell viability of RAW264.7 cells after stimulation with LPS in a dose dependent manner, with 10 μM C-DIM12 completely inhibiting the effects of LPS (Fig. 6c). Thus, 10 μM was selected as the therapeutic dose to be used in subsequent assays.

C-DIM12 suppresses LPS-stimulated NO production

To determine whether C-DIM12 suppressed production of NO in RAW264.7 cells, nitrite accumulation was measured in following stimulation with LPS. LPS stimulation alone induced NO production as expected, which increased over 24 hours (Fig. 7a). Conversely, cells pre-treated with 10 μM C-DIM12 had a significant suppression of NO after exposure to LPS for 24 hours (Fig. 7b).

C-DIM12 suppresses LPS-stimulated prostaglandin production

Arachidonic acid metabolites are autacoids and serve as extracellular and intracellular signals, which mediate nearly every step of the acute inflammatory response (Benedetti et al., 2014). These metabolites include prostaglandins such as PGE2, PGF2 and PGD2, which are produced by the COX-2 pathway. Given the central role of prostaglandin signaling in activated macrophages, PGD_2, PGE_2 and PGF_2 production by LPS-stimulated RAW264.7 cells was evaluated using LC-MS/MS. This assay revealed that LPS increased PGD_2, PGE_2 and PGF_2 within macrophage cells, while 10μM C-DIM12 treatment significantly suppressed the LPS-induced prostaglandin production (Fig. 8 a-c).

C-DIM12 inhibits LPS stimulated NF-κB, COX-2 and iNOS activation

Inflammation is mediated by a range of factors and cellular signaling pathways. Previous evidence has indicated that NF-κB dependent gene expression plays an important role in inflammatory responses and increases the transcription of genes encoding cytokines and pro-inflammatory mediators such as iNOS and COX-2 (Chen et al., 2003). Activation of NF-κB results in an increased expression of iNOS and COX-2 (Yamamoto et al., 2001; Aktan, F., 2004 and Cherukuri et al., 2014). iNOS, the enzyme responsible for NO synthesis, is closely associated with the cellular inflammatory response, as inflammation causes an overexpression of iNOS, thereby resulting the overproduction of NO (Atkan et al., 2004). To investigate the mechanism which C-DIM12 suppresses LPS-stimulated inflammatory mediator production, flow cytometry was used to analyze the mean fluorescence intensity (MFI) effects of C-DIM12 on NF-κB activation. LPS stimulation significantly increased NF-κB p65 expression when compared with the control group. However, treatment with C-DIM12 (10μM) suppressed LPS-

induced NF-κB p65 degradation (Fig 11). To further investigate the anti-inflammatory mechanism of C-DIM12, COX-2 and iNOS activation were examined by flow cytometry and immunofluorence. LPS stimulation significantly increased expression of both COX-2 and iNOS as expected, which was substantially suppressed by treatment with 10μM C-DIM12 (Fig.9 and 10).

C-DIM12 does not prevent p65 translocation

TLR4 receptors are activated in macrophages by binding of LPS to TLR4 ligands. NF-κB resides in the cytoplasm, upon activation must relocate to the nucleus to function. A common target for several anti-inflammatory molecules such as C-DIM12 is the inhibition of p65 translocation. To evaluate this, we treated RAW264.7 macrophage with 1 μg/ml LPS and measured the nuclear translocation of p65 using immunofluorescence. After an hour, p65 levels in RAW264.7 macrophage cells were increased over the control (Figure. 11B), as shown by the disappearance of nuclear voids evident prior to LPS treatments. Treatment with C-DIM12 did not inhibit the nuclear translocation of p65 in LPS stimulated RAW264.7 macrophage cells but significantly increased the nuclear levels of p65 (Figure 11A).

C-DIM12 Modulates Cellular Localization of Nurr1

We examined whether C-DIM12 altered expression of subcellular localization of Nurr1 in macrophages. RAW264.7 cells were treated with LPS (1μg/ml) or with LPS +C-DIM12 (10μM), and Nurr1 protein expression and localization was examined by confocal microscopy (Figure 12 B; Nurr1, red; DAPI, blue). Nurr1 expression was significantly increased over control in macrophage treated with LPS. Quantitation of fluorescence intensity of nuclear Nurr1 protein in macrophages treated with LPS + C-DIM12 (Figure 12A).

DISSCUSSION AND CONCLUSIONS

While the effects of C-DIM12 have been studied in neurodegeneration (De Miranda et al., 2015 and Hammond et al., 2015) and cancer models (Inamoto et al., 2008), little is known about its characteristics and behavior in peripheral macrophages. Results from this study show that C-DIM12 modulates LPS-induced pro-inflammatory mediators and signal transduction pathways in RAW265.7 murine macrophages. Specifically, C-DIM12 inhibited the upregulation and/or production of PGE_2, PGF_2, PGD_2, NO, COX-2 and iNOS following LPS stimulation. The production of inflammatory mediators NO, PG, iNOS, and COX-2 are mainly regulated by NF-κB. Our results additionally showed that C-DIM12 significantly inhibited LPS-induced NF-κB activation. Together, this data suggests that the C-DIM12 mediates inflammation in macrophages by modulating NF-κB signaling pathways. The observed increase in nuclear p65 after LPS+C-DIM12 treatment may be indicative of an increased amount of Nurr1 bound to p65, causing accumulation om the nucleus. Finally, we observed the translocation of Nurr1 from the cytosol to the nucleus in RAW264.7 macrophage treated with LPS occurred in the presence and the absence of C-DIM12.

In this study, we have conclusively shown that C-DIM12 inhibits several pro-inflammatory enzymes in RAW 264.7 cells and, in particular, suppresses NF-kB modulated pathways. Subsequent down-regulation of COX-2 and iNOS signaling also contributes to the anti-inflammatory actions of C-DIM12 and the decreased production of PGE_2 is a downstream consequence of these anti-inflammatory actions. Furthermore, increased nuclear p65 translocation and Nurr1 nuclear protein expression demonstrates a potential molecular target. These studies provide a molecular basis for utilizing C-DIM12 as an anti-inflammatory agent within pharmaceutical regimens to combat diseases and pathology associated with inflammation.

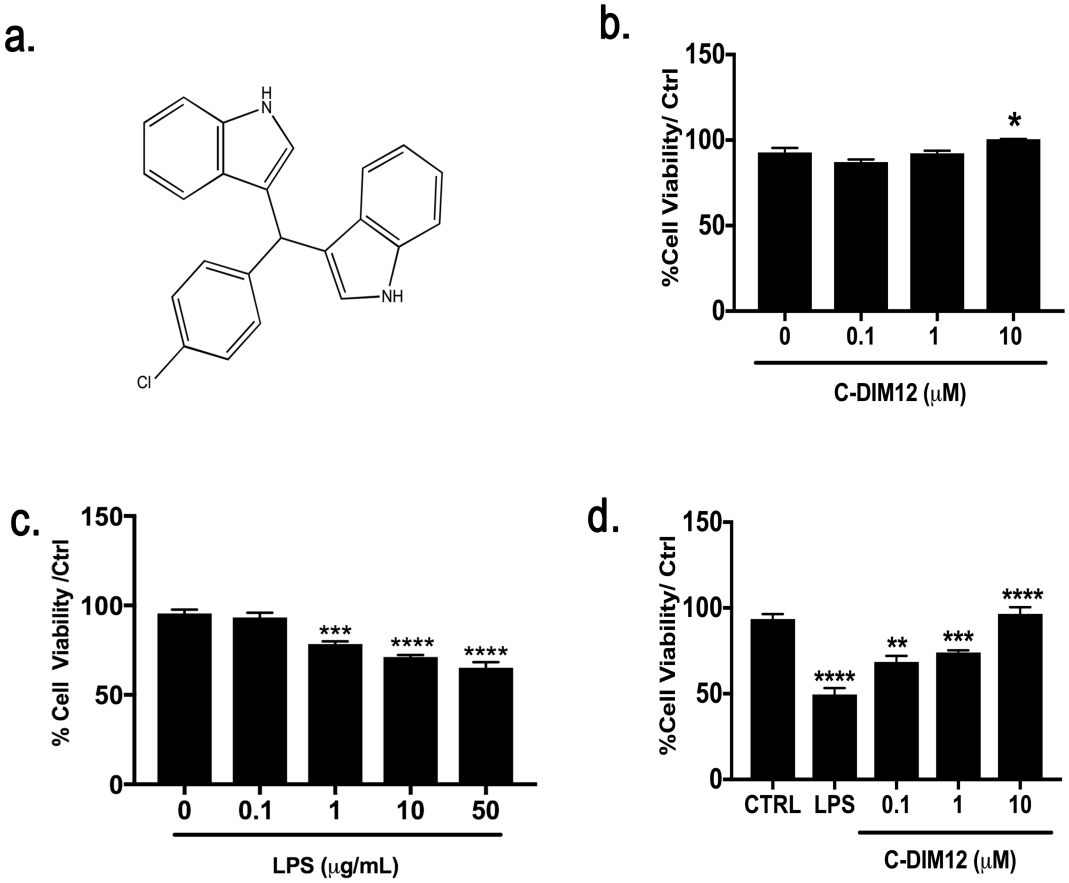


Figure 6. Effects of C-DIM12 on cell viability of RAW264.7 cells (a) The chemical structure of C-DIM12 and its effect on cell viability were measured by MTT assay (b) with C-DIM12 (0, 0.1,1and 10 μM) for 24 hr. (c) cell viability with LPS (0, 0.1, 1, 10, 50 μg/ml) for 24hr.(d) following treatment with or without 1μg/ml LPS and C-DIM12 (0, 0.1,1 and 10μM) for 24hr. Data were presented as the means ± S.E.M of four independent experiments. Statistical significance is compared with control and LPS stimulated control. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P< 0.0001$.

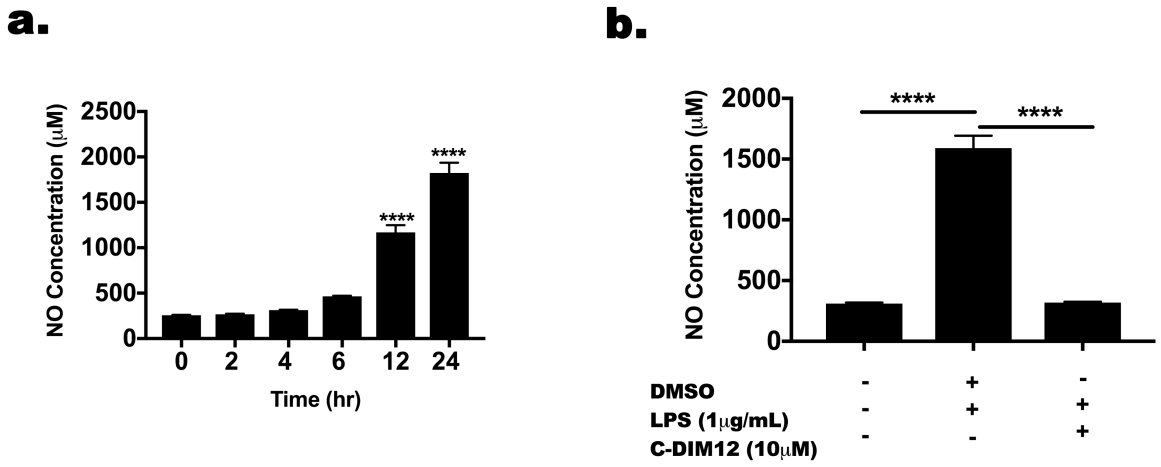


Figure 7. Effects of C-DIM12 on LPS-stimulated NO production (a) Quantitation of NO production in cells treated with LPS for varying duration of time (0-24 hrs). (b) Cells were treated with LPS with or without C-DIM12 for 24 hrs. Data were presented as the mean ± S.E.M of four independent experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P< 0.0001$ indicated significant difference from the LPS-stimulated group

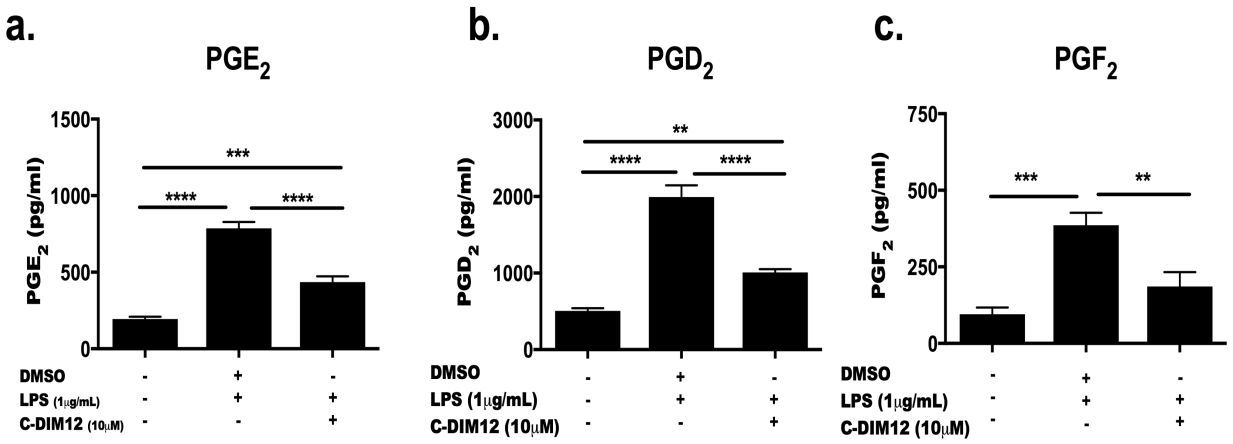


Figure 8. Effects of C-DIM12 on LPS-stimulated prostaglandin production (a-c). LC-MS/MS allows detection and quantification of the different classes of prostaglandins (PGE_2, PGF_2, and $PGD_{2)}$, Data were presented as the means ± S.E.M of three independent experiments after 24 hours of LPS treatment (Fig. 2b). * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P< 0.0001$ indicated significant difference from the LPS-stimulated group.

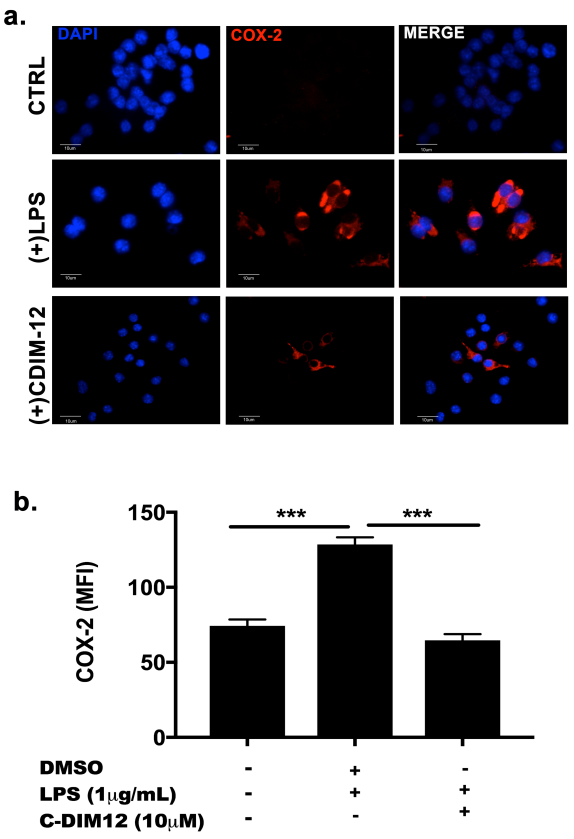


Figure 9. Effects of C-DIM12 on LPS stimulated COX-2 expression (a). Representative immunofluorence images of COX-2 with pretreatment of C-DIM12 (10 μM) followed by LPS (1μg/ml) stimulation (b). COX-2 MFI expression was determined by flow cytometry, over 5,000 events from intact non-aggregate cells. Data is expressed as means ± S.E.M of four independent experiments. * P<0.05, ** P<0.01, *** P<0.001, **** P< 0.0001 indicated significant difference from the LPS-stimulated group.

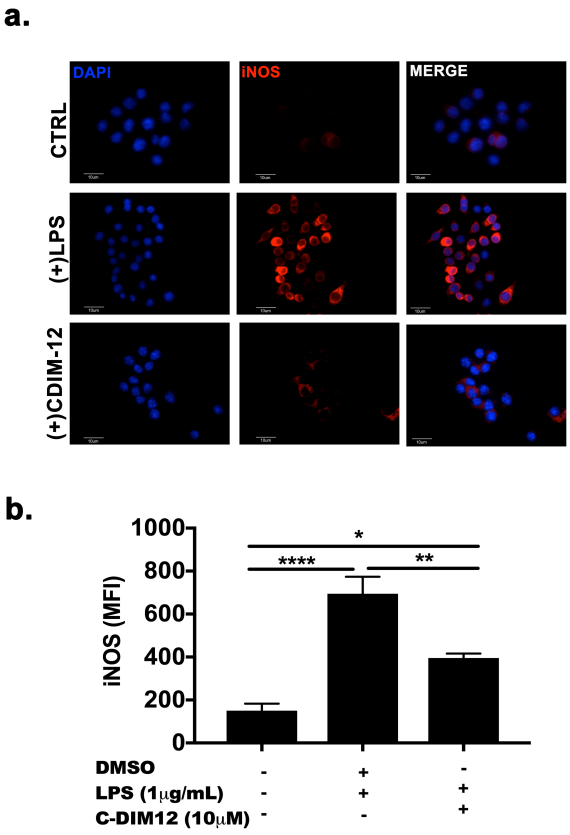


Figure 10. Effects of C-DIM12 on LPS stimulated iNOS expression (a). Representative immunofluorence images of iNOS expression with pretreatment of C-DIM12 (10 μM) followed by LPS (1μg/ml) stimulation (b) iNOS MFI expression was determined by flow cytometry, over 5,000 events from intact non-aggregate cells. Data is expressed as means ± S.E.M of four independent experiments. * P<0.05, ** P<0.01, *** P<0.001, **** P< 0.0001 indicated significant difference from the LPS-stimulated group.

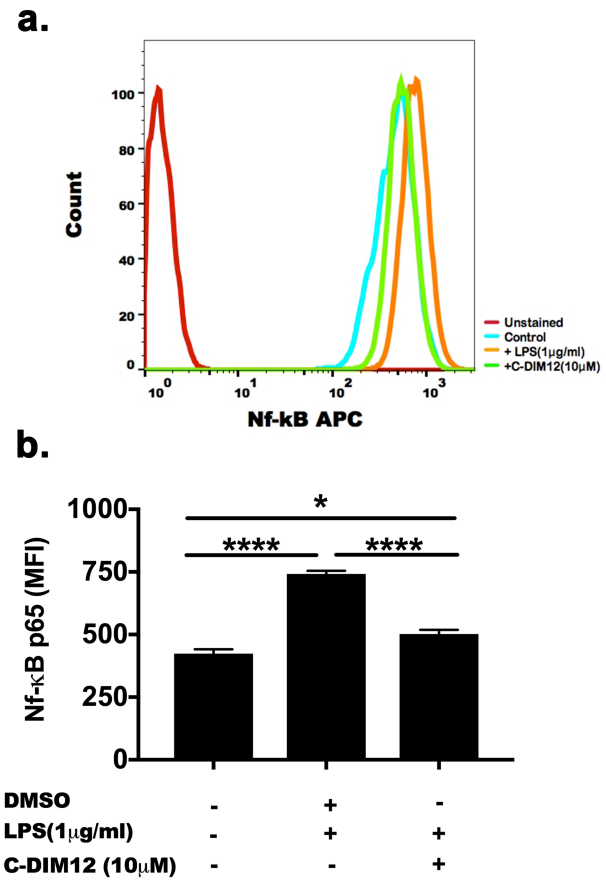


Figure 11. Effects of C-DIM12 on LPS stimulated NF-κB expression (a). Overlaid histogram showing Nf-κB expression with or without LPS (1μg/ml) stimulation and pretreatment of C-DIM12 (10 μM) followed by LPS (1μg/ml) stimulation (b). Nf-κB MFI expression was determined by flow cytometry over 5,000 events from intact non-aggregate cells. Data is expressed as means ± S.E.M of four independent experiments. * P<0.05, ** P<0.01, *** P<0.001, **** P< 0.0001 indicated significant difference from the LPS-stimulated group.

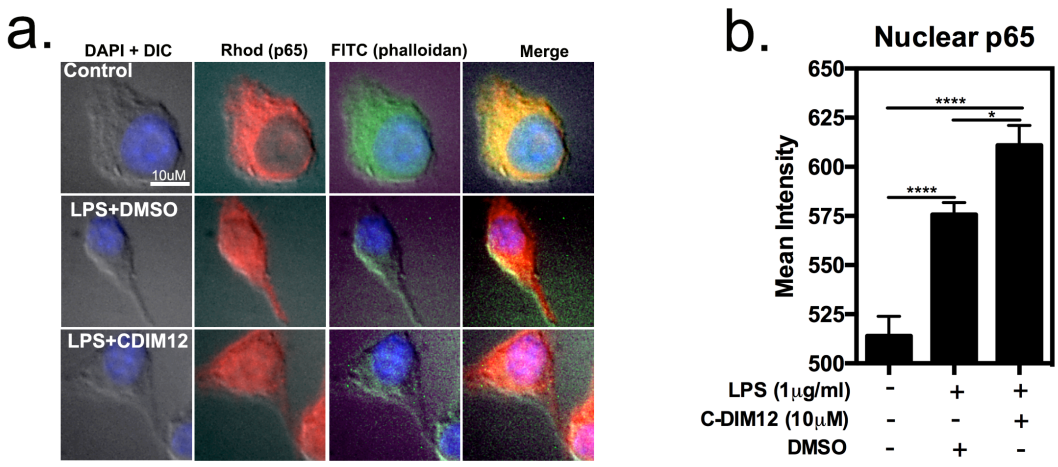


Figure 12. C-DIM12 does not prevent p65 translocation (a). RAW264.7 cells were treated with C-DIM12 (10 μM) followed by LPS (1μg/ml) for 1 hr and fixed for immunofluorescence to examine p65 translocation with DAPI (blue), phallodian (green), p65 (red). (b) The p65 nuclear expression was quantified by mean fluorescence intensity encompassing the nuclei (DAPI boundary; background subtracted). Data is expressed as means ± S.E.M of four independent experiments. * P<0.05, ** P<0.01, *** P<0.001, **** P< 0.0001 indicated significant difference from the LPS-stimulated group.

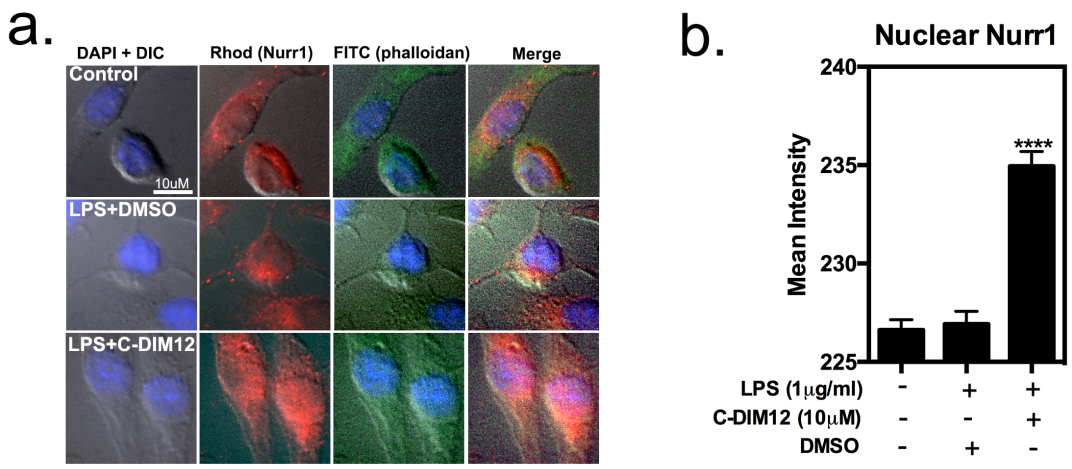


Figure 13. C-DIM12 Modulates cellular localization of Nurr1 (a). RAW264.7 cells were treated with C-DIM12 (10 μM) followed by LPS (1μg/ml) for 1 hr and fixed for immunofluorescence to examine Nurr1 with DAPI (blue), phallodian (green), Nurr1 (red). (b) Nurr1 protein translocation from the cytoplasm to the nucleus was quantified by mean fluorescence intensity encompassing the nuclei (DAPI boundary; background subtracted). Data is expressed as means ± S.E.M of four independent experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P< 0.0001$ indicated significant difference from the LPS-stimulated group.

CHAPTER 4

A NOVEL PARA-PHENYL SUBSTITUTED DIINDOLYLMETHANE INHIBITS INFLAMMATORY RESPONSE IN MURINE SYNOVIAL FIBROBLASTS

INTRODUCTION

Rheumatoid arthritis (RA) has a prevalence of 1-2% and is one of the more common causes of chronic morbidity among people over 65 years of age (Ping et al., 2000). It is characterized by hyperplasia of fibroblast-like synovial cells (FLS) within the synovium and recruitment of multiple leukocyte populations that drive the inflammatory process (Hardy et al., 2013). Although disease etiology is unknown, it is thought that both genetic and environmental factors trigger the onset of RA (Veale et al., 2002). The onset of RA is clinically recognized when a patient presents symptoms fulfilling disease classification criteria brought forth by the American College of Rheumatology criteria for RA. However, it is recognized that disease onset may occur much earlier, even prior to symptom onset (Ardle et al., 2015). The disease course of RA can range from mild and self-limiting to severe and progressive.

RA synovial fibroblasts represent a unique cell type that distinguishes RA from other inflammatory conditions of the joints. Pro-inflammatory mediators including tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1 are released by macrophage cells to promote synoviocyte cell proliferation including the release of matrix-degrading enzymes with the induction of pro-angiogenic factors and secretion of cytokines and chemokines (Aherne et al., 2009). These Proinflammatory cytokines are master regulator of chronic inflammation and tissue

destruction in RA. In response to these cytokines, FLS produce chemokines, and adhesion molecules that further promote inflammation, hyperplasia, and cartilage destruction (Choy and Panayi, 2001; Iwamoto et al., 2008; Jones et al., 2013). However, several lines of evidence have indicated that synovial fibroblasts can also become activated under disease conditions, and can contribute to inflammatory damage at the joint.

Previous studies have established that within the cytokine network in RA, TNF-α is a critical effector in the proinflammatory cytokine cascade (Feldman et al., 1996). The family of nuclear factor-kappaB (NF-κB) Transcription factors is significantly involved in the regulation of expression of numerous genes in the inflammatory response (Sweeney, S.E. and Firestein, G.S., 2004). NF-κB has been identified as a B-cell specific factor binding to specific DNA sequences within the enhancer element of light-chain immunoglobin genes (Barnes, P.J. and M. Karin, 1997). NF-κB consists of hetero- and homodimers that can include p50 and/p65 subunits. The complex normally resides in the cytoplasm of cells and is suppressed by a constitutively produced inhibitor known as IκB. (Tabas, I., and Glass, C., 2013). Following activation by various stimuli, including cytokines and LPS. IκB is phosphorylated and degraded by the ubiquitin-proteasome system. The released NF-κB then translocates to the nucleus where it binds to specific NF-κB DNA binding sites and initiates gene expression. NF-κB is a critical transcription factor involved in production of many inflammatory cytokines and adhesion molecules (De Miranda et al., 2014).

To determine the expression and the regulation of NF-κB in inflammatory arthritis, we assessed adhesion molecules and transcription factors expressed in stimulated primary synovial fibroblasts.

MATERIALS AND METHODS

Reagents. C-DIM12 was synthesized by Dr. Stephen Safe and characterized as described by Qin et al. (2004). All general chemical reagents were purchased from Sigma, Aldrich (St. Louis, MO) unless stated otherwise. TNFα was purchased from R&D Systems (Minneapolis, MN). Antibodies VCAM-1, ICAM-1, Cd11b, CD90.2, COX-2, Vimentin, Iκβα, Nurr1 and p65 used in flow cytometry and immunofluorescences experiments were purchased from BD Biosciences (San Jose, CA), Santa Cruz Biotechnology (Dallas, TX), and Cell Signaling Technology (Danvers, MA) respectively.

Primary Cell Isolation. Primary synovial fibroblasts were isolated from the ankles from C57/Bl6 6- 8 weeks old mice (unknown genders) according to procedures described (Armaka et al., 2009) and purity was confirmed through FACS and Immunofluorescent staining using antibodies against CD90.2, Cd11b, VCAM-1, ICAM-1 and vimentin. In brief, 6-8-week-old mice were euthanized using carbon dioxide, hind legs were rapidly dissected out with the foot intact. Tissue was subjected to digestion with collagenase from Clostridium hitolyticum type IV (1mg/ml). This method routinely results in cultures that are approximately 99% pure synovial fibroblasts with less than 1% contaminating macrophage cells (figure 1). Synovial fibroblasts cultures were maintained in Dulbecco's modified Eagle's media supplemented with 10% FBS, and 1x PSN. The cells were maintained at 37°C with 5% CO_2. Synovial fibroblasts were used up to passage 3 of plating. All procedures involving animals were approved by the Colorado State University Institutional Animal Care and Use committee and were conducted in accordance with current National Institutes of Health guidelines.

Gene Knockdown Assay. RNA interference (siRNA, small interfering RNA) sequences were obtained through Integrated DNA technologies (IDT DNA, Coralville, IA). Nurr1 RNAi duplexes were designed against splice common variants of Nurr1 and were validated using a dose response assay with increasing concentrations of the suspended oligo (1.2 μg and 1.5 μg) using a standard scrambled dicer- substrate RNA as control. RNAi oligos were transfected using the T*rans*IT-X2 delivery system (Mirus Bio, Madison, WI) for 24 hours. Separate siRNA systems were used to ensure specific knockdown of Nurr1 mRNA. The Nurr1 DsiRNA duplex sequences (5'->3') CUAGGUUGAAGAUGUUAUAGGCACT; AGUGCCUAUAACAUCUUCAACCUAGAA (IDT DsiRNA, denoted RNAi).

Enzyme-linked Immunosorbent assay. Primary mouse synovial fibroblasts were untreated and treated with TNF-α (0-100 ng/ml) for 12 h prior to harvesting conditioned media. Primary synovial fibroblasts were also untreated and treated with 10 μM C-DIM12 followed by 10ng/ml TNFα for 12 h prior to harvesting conditioned media. The conditioned media was preserved with 1% BHT solution to avoid the free-radical peroxidation as explained in (Cao et al., 2008). Samples were stored at -20°C until analysis. PGE_2 analysis and quantification was conducted via ELISA using a PGE_2 analysis kit (R&D Systems) in accordance with the manufacturer's instructions, except samples were not diluted as indicated. The experiment was conducted three independent experiments run in triplicates.

FACS analysis. Synovial fibroblasts (3×10^5 per well) were plated in 6 well plates and allowed to adhere for 18 hrs. The cells were then pretreated with for 1 hr. with 10 μM C-DIM12 followed by 10ng/ml TNFα for 12 hrs. The cells were then harvested and fixed with 2%

paraformaldehyde for 10 mins. Cells membranes were permeabilized with 0.01% triton X-100 in PBS for 30 mins and stained with antibodies against NF-κB, COX-2, Nurr1 and IκBα for 1 hr. at 37 °C. Secondary incubation was at 37 °C for 1 hr. with Alexa flour 647. Unstained cells were used as negative controls during acquisition. Flow cytometry was conducted on a Beckman Coulter CyAn^{ADP} flow cytometer operating with Summit v4.3 software for data collection. All further analysis was done with FlowJo (version 10.0.8; Software, Ashland, OR). Samples were run in biological triplicates with two technical duplicates.

Cytokine/chemokine determination in cell culture supernatants by Cytometric Bead Array (CBA). Supernatant was collected and immediately processed for Interleukin-6 (IL-6), interleukin-10 (IL-10), monocyte Chemoattractant protein-1 (MCP-1), and interferon-γ (IFN-γprotein levels were quantitatively measured by BD CBA Mouse Inflammation Kit (BD Biosciences, San Jose, CA). The protocol was performed according to the manufacturer's instructions. The intensity of fluorescence signal was acquired on a Beckman Coulter CyAn^{ADP} Flow cytometry operating with Summit v4.3 software for data collection. All further analysis was done with FlowJo (version 10.0.8; Software, Ashland, OR). Samples were run in biological triplicates with two technical duplicates.

Statistical analysis. Statistical analyses were performed using Prism (version 7.0; Graph Pad Software, San Diego, CA). Data are presented as mean ± S.E.M. Experimental group analyses were performed using a one-way analysis of variance with a Tukey post hoc test. *$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$, **** $P< 0.0001$ were considered statistically significant.

RESULTS

Generation of primary synovial fibroblast cultures.

Primary synovial fibroblasts were isolated by enzymatic digestion of the ankle joints of C57/Bl6 6- 8 weeks old mice. A homogenous cell population characteristic of synovial fibroblasts was subjected to FACS analysis on passage 3 after seeding to verify purity. Cells were stained for their original characteristics of constitutive CD90.2, VCAM-1 and ICAM-1 expression. Immunofluorescent confirmed Vimentin expression, a mesenchymal marker. As seen in Figure 1, CD90.2 has been shown to be >80-90% of the cell populations whereas Cd11b a monocyte marker confirmed <1% of the population. Therefore, confirming purity of the murine synovial fibroblasts isolation (Figure 12).

Constitutive and TNF-α induced VCAM-1 and ICAM-1 expression on synovial fibroblasts.

Binding of TNF-α to adhesion molecules such as VCAM-1 and ICAM-1 in synovial fibroblasts activates the inflammatory response; this interaction is critical in the involvement of recruitment and retention of immune cells in the inflamed joints. VCAM -1 expression, including TNF-α responsiveness, was determined in this cell population (Figure 13). Although VCAM-1 is constitutively expressed, its surface levels are significantly increased after exposure to 10 ng/ml of TNF-α for 12 h (Figure 13a). The response was dose-and time dependent (Figure 13, a and b) with maximal level detected after stimulation with 100 ng/ml for 12 h. We next examined the effect of C-DIM12 in suppressing VCAM-1 and ICAM-1 expression by treating synovial fibroblasts with TNF-α (10 ng/ml) for 12 hours in the presence of C-DIM12 (10μM). Adhesion molecules expression was significantly decreased over TNF-α and vehicle control (DMSO) expression levels with 10μM of C-DIM12 (Figure 13, c and d).

C-DIM12 Decreases Inflammatory PGE_2 Expression in Synovial Fibroblast.

Exposure of synovial fibroblast to TNF-α caused production of PGE_2 increase in a dose dependent with the maximal dose of 100 ng/ml for 24-hour exposure (Figure 14a). To further examine the effect of C-DIM12 on PGE_2 production, we exposed synovial fibroblasts to TNFα in the absence and presence of C-DIM12. Co-treatment with C-DIM12 significantly reduced PGE_2 production (Figure 14b).

C-DIM12 Decrease COX-2 Expression in Synovial Fibroblasts.

Since, C-DIM12 could inhibit TNF-α induced production of PGE2, we further examined effects of C-DIM12 on COX-2 expression. As shown in figure 3, COX-2 protein was significantly lower in resting synovial fibroblasts and highly induced in the presence of TNF-α. C-DIM12 significantly inhibited COX-2 production back to resting state of synovial fibroblasts (Figure 15).

C-DIM12 Decrease IκBα and p65 NF-κB Expression in Synovial Fibroblasts

Phosphorylation and degradation of IκBs by IKK lead to nuclear translocation of Rel family proteins and activation of NF-κB occurs. To elucidate inhibitory mechanism of NF-κB activation, we explored cytoplasmic levels of IκBα and p65 NF-κB. Flow Cytometry analysis revealed that IκBα protein expression decreased significantly with treatment of C-DIM12 (Figure 16), and resulted in a decrease of IκBα phosphorylation. Next, protein expression of stimulated TNF-α synovial fibroblasts were analyzed for NF-κB p65 using flow cytometry. Amount of protein of NF-κB p65 was markedly increased upon exposure to TNF-α (Figure 16); this effect was inhibited by C-DIM12.

C-DIM12 dependent Inhibition of Inflammatory gene expression requires Nurr1.

We used RNAi to examine the capacity of C-DIM12 to function as a modulator of Nurr1 through its effects of NF-κB regulated inflammatory genes. The data shown in Figure 6A indicated that treatment of synovial fibroblasts with two different concentration of siRNA against Nurr1 resulted in significant knockdown of Nurr1 mRNA expression. Showing more significant knockdown occurring with a lower concentration of siRNA. After RNAi knockdown of Nurr1, synovial fibroblasts were treated with TNF-α in both the presence and absence of C-DIM12 (10 μM) for 24 hours and then assayed via flow cytometry for Nurr1 expression (Figure 17b). In the group receiving RNAi, C-DIM12 no longer suppressed the TNF-α induced expression of Nurr1. Synovial fibroblasts were further analyzed for expression of NF-κB regulated inflammatory genes IL-6, MCP-1 and two other inflammatory genes implicated within the inflammatory response of RA IL-10 and IFN-γ (Figure 17 c-f). Once again, we found the treatment groups receiving Nurr1 RNAi were no longer suppressed with C-DIM12 after the TNF-α induced expression of IL-6, MCP-1, IL-10 and IFN-γ. These data support that C-DIM12 requires suppression of Nurr1 to fully suppress pro-inflammatory gene expression in TNF-α induced synovial fibroblast.

DISSCUSSIONS AND CONCLUSIONS

RA is an inflammatory disease where the pathology is both triggered and sustained by the influx of expression of cytokines, which are responsible for the destruction of the joint (Li, P., et al., 2000). Pro-inflammatory cytokines that are overexpressed in RA joints, have been shown to induce a variety of disease genes, including other cytokines, proteases, oxygenases and adhesion molecules, in addition to stimulating cell proliferation (Feldmann, M.,

et al., 1996). Pro-inflammatory cytokines such as TNF-α has been extensively been researched because of its involvement within the pathogenesis of RA. TNF-α mediates the cellular infiltration of the joint while the development of the pannus is responsible by white blood cells releasing cytokines that prompt the synovial membrane's blood vessels to multiply; therefore, causing an increased blood flow leading to excess tissue growth causing the synovial cells to reproduce at an abnormal increased rate and resulting in the thicken of the synovium. Thus, the cellular pathway involved in the induction of VCAM-1 by TNF-α in primary synovial fibroblasts is a target for alternative therapeutic. In this study, we investigated the regulation of NF-κB in inflammatory arthritis, we assessed adhesion molecules and transcription factors expressed in stimulated primary synovial fibroblasts.

Previous studies have shown the structure-dependent binding of some C-DIMs to the ligand-binding domain of NR4A1 (Lee et al., 2014) and these compounds can either activate or inactivate NR4A1 and NR4A2 dependent transactivation specific to the structure (Cho et al., 2010; Yoon et al., 2011; Lee et al., 2014; De Miranda et al., 2015).

The data presented here indicates that C-DIM12 suppresses NF-κB induced gene expression in synovial fibroblasts through a mechanism involving transcriptional repression of Nurr1 (Figure 17). Dose dependent inhibition of adhesion molecules in synovial fibroblasts indicate that C-DIM12 blocks expression of the initiation of inflammatory response (Figure 13). In addition, C-DIM12 suppressed of PGE2 production and COX-2 expression in TNF-α stimulated synovial fibroblasts (Figures 14 and 15). C-DIM12 suppressed IκBα protein expression therefore resulted in a decrease of IκBα phosphorylation. Lastly, C-DIM12 significantly decreased NF-κB p65 expression in stimulated TNF-α synovial fibroblasts.

In conclusion, these experiments provide evidence that NF-κB directly mediates the induction of VCAM-1 in synovial fibroblasts by TNF-α and furthermore C-DIM12 suppresses this activation. Demonstrating that C-DIM12 induces activity in synovial fibroblasts through a Nurr1 dependent mechanism. Providing a novel mechanism to decrease expression of NF-κB regulated inflammatory genes in synovial fibroblast cells relevant to degenerative joint diseases.

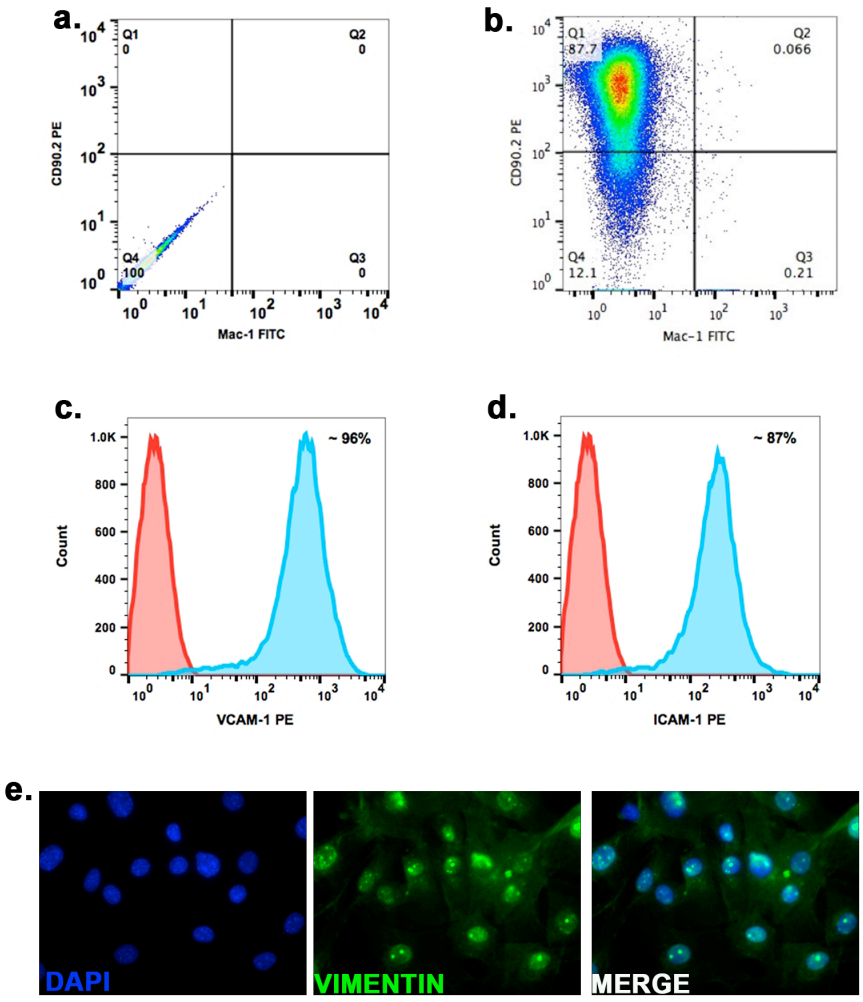


Figure 14. Immunophenotype of Primary Synovial Fibroblasts. Isolated synovial fibroblasts from WT mice were subjected to FACS analysis on passage 3 after seeding to verify purity. (a) negative control for dual stain cells (b)Density plot showing expression of CD90.2 on the majority of the isolated cells (>90 %) whereas very few Mac-1 positive myeloid cells can be detected (<1%). (c) Overlaid histogram showing VCAM-1 (~96%) (d) ICAM-1 expression (86%) (blue shaded area) on SFs or unstained control (red shaded area).(e) representative image for Vimentin expression in synovial fibroblasts.

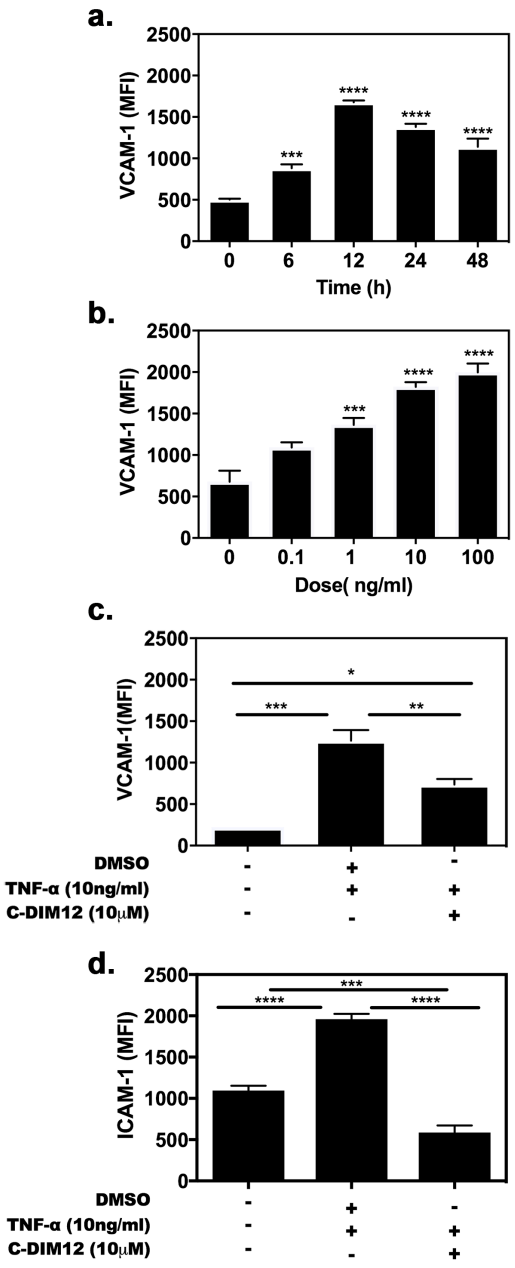


Figure 15. Constitutive and TNF-α induced VCAM-1 and ICAM-1 expression on synovial fibroblasts. (a) Primary Synovial Fibroblasts were cultured in a dose dependent manner with TNF-α (10 ng/ml) for 12hr. And then VCAM-1 surface expression was determined by FACS, as described in Methods. (B) The dose response of VCAM-1 expression was determined 12 h after TNF-α stimulation. (C) The time course of VCAM-1 expression was performed by stimulating the cells with TNF-α (10ng/ml). (c, d) synovial fibroblasts were stimulated with TNF-α (10ng/ml) treated with 10μM CDIM12 analyzed for Data are expressed as mean ± S.E.M (n=4). Statistical significance is compared with saline control. *P < 0.05, **P<0.01, ***P<0.001, ****P<0.0001.

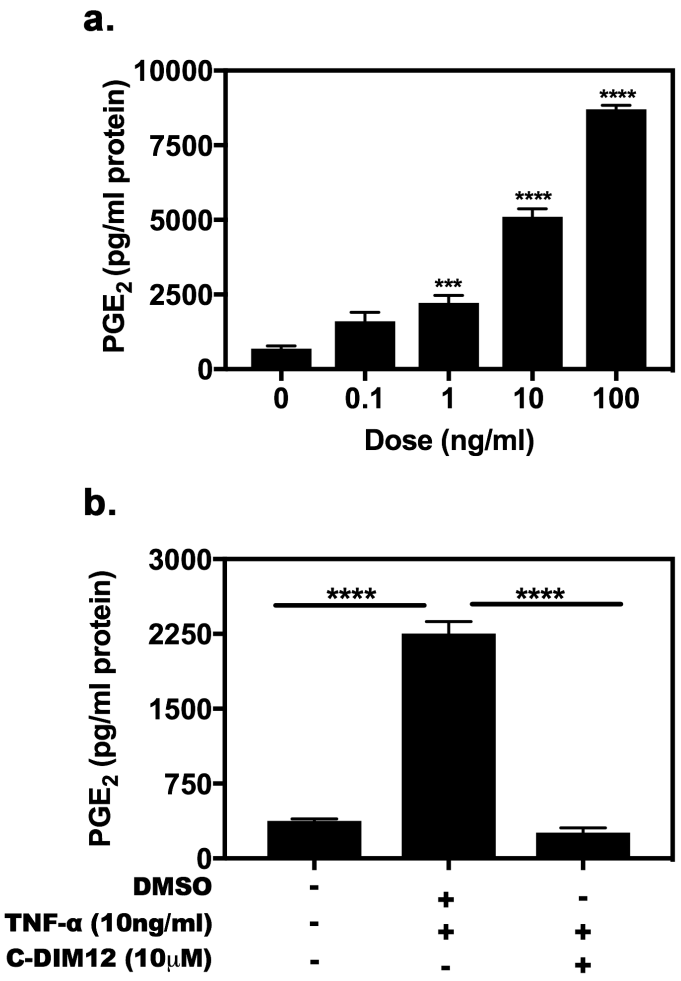


Figure 16. C-DIM12 Decreases Inflammatory PGE_2 Expression in Synovial Fibroblast. (a) The dose response was determined 24 hours after TNF-α (10ng/ml) stimulation (b) TNF-α (10ng/ml) stimulated treated with 10μM CDIM12. Data are expressed as mean ± S.E.M (n=4). Statistical significance is compared with saline control. *P < 0.05, **P<0.01, ***P<0.001, ****P<0.0001.

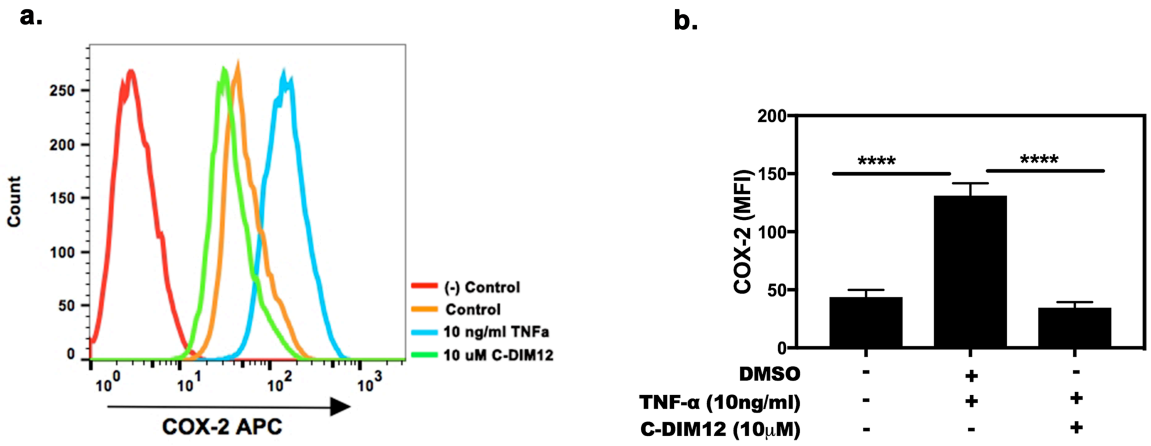


Figure 17. C-DIM12 Decreases COX-2 Expression in Synovial Fibroblasts. (a) Overlaid histogram showing COX-2 expression stimulated synovial fibroblast expression increase shift to right and followed by treatment of C-DIM12 (10 μM) decrease back to basal condition. (b) Synovial fibroblasts were stimulated with TNF-α (10ng/ml) treated with 10μM CDIM12 analyzed for COX-2 expression. Data are expressed as mean ± S.E.M (n=4). Statistical significance is compared with saline control. *P < 0.05, **P<0.01, ***P<0.001, ****P<0.0001.

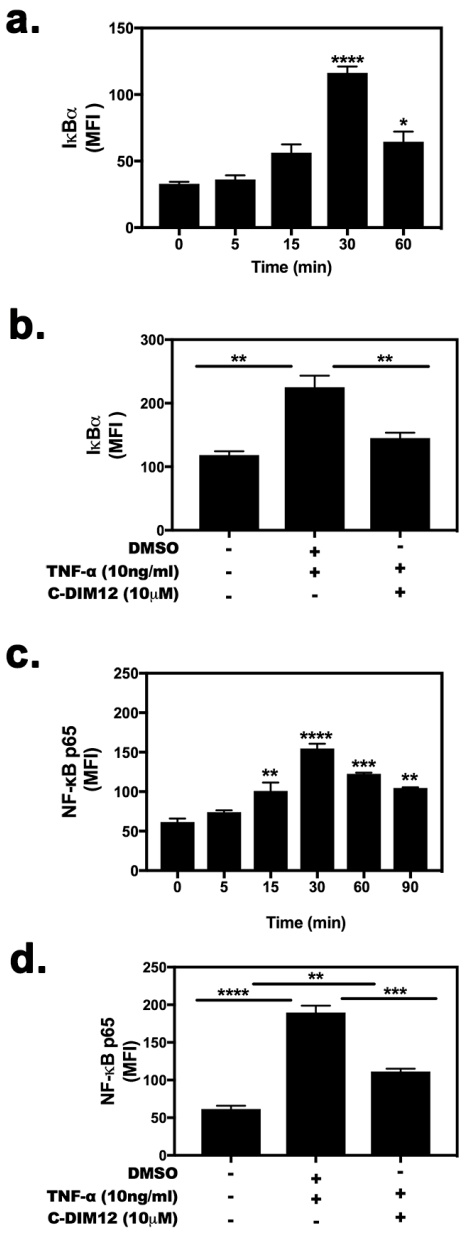
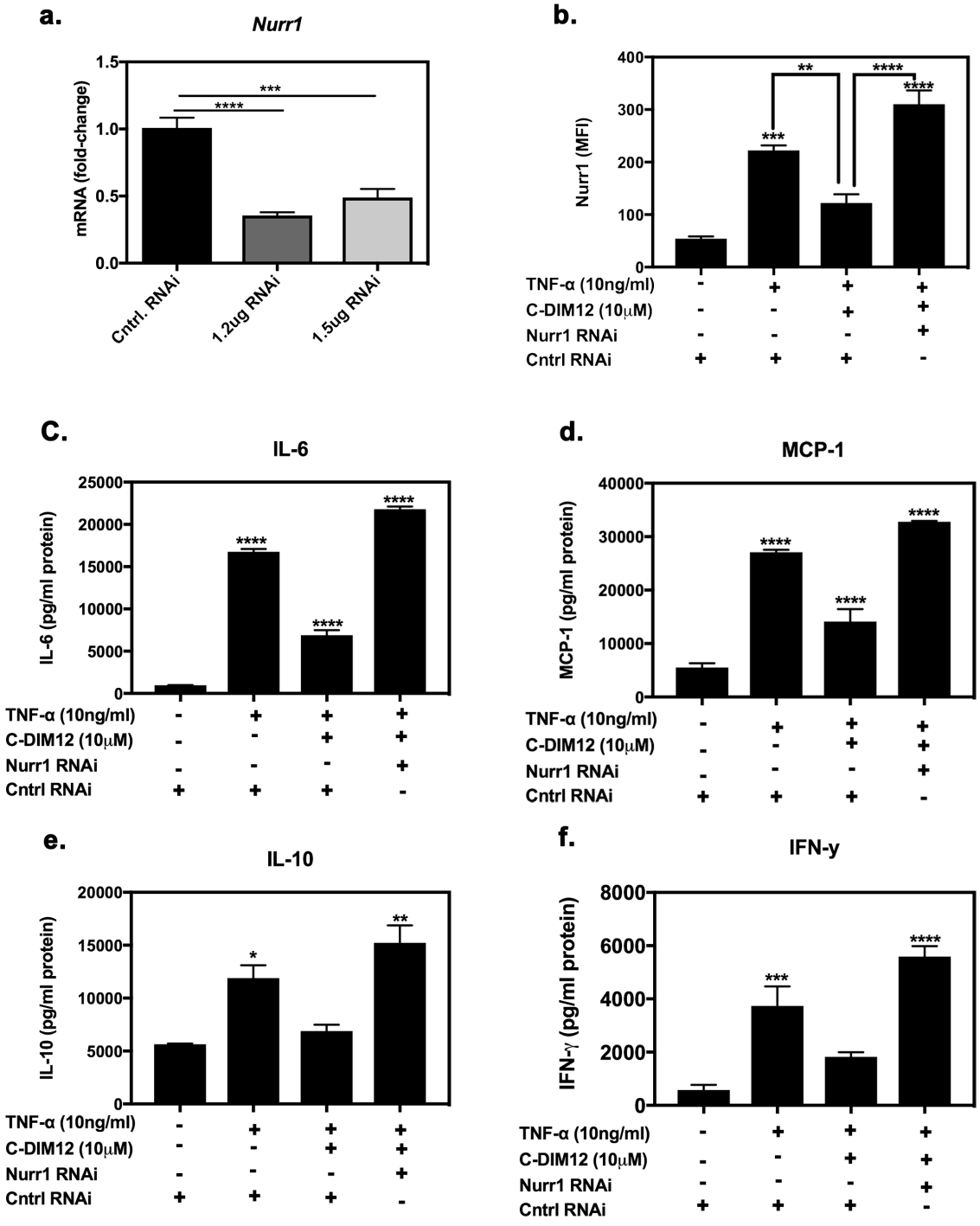


Figure 18. C-DIM12 Decrease IκBα and p65 NF-κB Expression in Synovial Fibroblasts. (a and c) The dose response of IκBα and NF-κB expression was determined 30 mins after TNF-α (10 ng/ml). (b and d) synovial fibroblasts were stimulated with TNF-α (10ng/ml) treated with 10μM CDIM12 analyzed by flow cytometry. Data are expressed as mean ± S.E.M (n=4). Statistical significance is compared with saline control. *P < 0.05, **P<0.01, ***P<0.001, ****P<0.0001.


a.
Nurr1
mRNA (fold-change)
1.5
1.0
0.5
0.0
***
****
Cntrl. RNAi
1.2ug RNAi
1.5ug RNAi
b.
Nurr1 (MFI)
400
300
200
100
0
**
****
***
****
TNF-α (10ng/ml) - + + +
C-DIM12 (10μM) - - + +
Nurr1 RNAi - - - +
Cntrl RNAi + + + -
C.
IL-6
IL-6 (pg/ml protein)
25000
20000
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TNF-α (10ng/ml) - + + +
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Nurr1 RNAi - - - +
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TNF-α (10ng/ml) - + + +
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Nurr1 RNAi - - - +
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IL-10
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TNF-α (10ng/ml) - + + +
C-DIM12 (10μM) - - + +
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TNF-α (10ng/ml) - + + +
C-DIM12 (10μM) - - + +
Nurr1 RNAi - - - +
Cntrl RNAi + + + -

Figure 19. C-DIM12 dependent inhibition of inflammatory gene expression requires Nurr1. (a) Synovial fibroblasts were treated with two concentrations of siRNA denoted RNAi) or scrambled RNAi (control RNAi). (b) Synovial fibroblasts were treated with Nurr1 RNAi or control RNAi for 24 hours followed by saline or 10 ng/ml TNF-α for 24 hours and assessed for Nurr1 expression via flow cytometry. (c-f) Synovial fibroblasts were treated with Nurr1 RNAi or control RNAi for 24 hours followed by saline or 10 ng/ml TNF-α for 24 hours and assessed for IL-6, MCP-1, IL-10 and IFN-γ protein concentration via flow cytometry. Data are expressed as mean ± S.E.M (n=4). Statistical significance is compared with saline control. *P < 0.05, **P<0.01, ***P<0.001, ****P<0.0001.

CHAPTER 5

APPLICATIONS OF FLOW CYTOMETRY FOR GLIAL CELL CULTURES FROM MOUSE BRAIN

INTRODUCTION

Traditionally, flow cytometry has been used to identify distinct cell types within a heterogeneous pool of cells based on extracellular or surface marker expression, commonly known as immune-phenotyping. The ability to simultaneously measure multiple parameters on a cell-by-cell basis is probably the most powerful aspect of flow cytometry. This allows flow cytometry to be used for a wide range of applications, including detection of intracellular targets and complex signaling events. Here, we provide a detail description of protocols for flow cytometric analysis of Glial cell populations. These applications include immunophenotyping of primary mixed glial cultures, purification of microglia and astrocytes, and apoptosis assay.

Astrocytes are morphologically characterized by their classic expression of the intermediate filament proteins glial fibrillary acidic protein (GFAP) and Vimentin. Other known markers of astrocytes in adult brain include glutamine synthetase (GS), S100 calcium binding protein, and glutamate transporters GLT-1/EAAT1 and GLAST/EAAT1 (Kimemlberg, 2004). Here we stained cultured astrocytes for the presence of GLAST/EAAT1.

As the resident macrophage in the central nervous system (CNS), microglia play an important role in the response of the brain to both foreign and endogenous insults. Pioneering antibody research in the 1990's identified the Integrin beta 2 protein (also called ITGB2, complement receptor 3,CR3, CD18, and Mac-1) as a key protein in the recruitment of microglial

cells. The ITGB2 subunit A is commonly called CD11b, and antibodies to this subunit are widely used as microglial markers (Bennett et al., 2016).

In addition to these Immuno-phenotyping assays, an apoptosis study was conducted to investigate Neuro-2A (N2A) cells response when treated with astro-conditioned media (ACM) or glia-conditioned media (GCM or microglia (micro)-conditioned media (MCM)) after glia were treated for 48 hours with $MnCl_2$. Flow cytometry was used to assess N2A apoptosis using Annexin-V and Propidium Iodide (PI) viability staining upon GCM exposure for 48 hrs.

MATERIALS AND METHODS

Cell culture. Mixed glial cultures from whole brain (excluding cerebellum and brain stem) were prepared from 1-day-old transgenic mice expressing an enhanced-green fluorescent (EGFP) reporter under the control of three cis NF-κB elements (*cis*-NF-κB^{EGFP}; C57Bl6/J background;(Magness, S.T., et al., 2004, generously provided by Dr. Christian Jobin, University of North Carolina at Chapel Hill) using a modification of a previously described method (Moreno, J.A., et al., 2008; Aschner, M. et al., 1991; Carbone et al., 2008) . Briefly, mice were euthanized by decapitation under isofluorane anesthesia and whole brains were rapidly dissected out and placed into ice-cold minimum essential medium with L-glutamine (MEM; Gibco/Invitrogen, Grand Island, NY). Meninges were removed and tissues completely digested with dispase (1.5U/ml; Gibco). Dissociated cells were plated onto 100-mm tissue culture plates and kept in Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated FBS (Sigma, St. Louis, MO) and penicillin (0.002 mg/ml), streptomycin (0.002 mg/ml), and neomycin (0.001 mg/ml) antibiotic mixture (PSN). Media was changed every 4-5 days and cells

were maintained at 37°C and 5% CO_2 in humidified chambers until cultures were confluent (~14-18 days).

Purification of Astrocytes and Microglia. Microglia were purified from astrocytes via column-free magnetic separation using the EasySep mouse CD11b positive selection kit (Stemcell technologies, Vancouver, Canada) according to manufacturer instructions and as outlined in Gordon, R.R. et al., 2011. Cells from confluent mixed glial cultures (~14-18 days old) were detached using 0.25% trypsin (Gibco). Trypsin reaction was halted using MEM complete media and remaining cells were removed using a cell lifter. Harvested cells were gently triturated and passed through a 70μm cell strainer to remove any cell aggregates. The cell mixture was then centrifuged and resuspended at 1 x 10^8 cells/ml in calcium- and magnesium-free phosphate buffered saline containing 2% FBS and 1 mM EDTA. Cells were transferred to a 5-ml round bottom tube and were incubated at room temperature with the CD11b-Phycoerythin (PE) monoclonal antibody (50μL/mL) for 15 minutes then EasySep PE-Selection Cocktail (70μL/ml) for 15 minutes followed by a 10 minute incubation with Dextran coated EasySep magnetic nanoparticles (50μL/ml). Cells suspension was brought up to 2.5 mL of media, gently mixed and placed in the EasySep magnet for 5 min to isolate immune-linked cells. After 5 min, the tube was inverted and the cell solution of un-labeled cells was collected while labeled cells remained in the tube. The solution remaining in the tube was resuspended in another 2.5mL of recommended media and placed in the magnet. This process was repeated for a total of 5 extractions. The purified microglia in the positive fraction and purified astrocytes in the final three negative fractions were resuspended in complete MEM and seeded onto respective tissue

culture plates. Purified astrocytes and microglia were utilized in experiments within a week of purification.

Flow Cytometry Immuno-phenotyping. The estimated percent of glia in mixed glial, microglial, and astrocyte cultures were determined by immunophenotying using direct labeling with anti-GLAST- PE (Miltenyti biotec, San Diego, CA), anti-Cd11b–FITC (BD Biosciences), anti-CD11b-PE (Stem Cell Technologies), and anti-GLAST-488 (Novus Biologicals, Littelton, CO) followed by flow cytometric analysis. Cells were counted using a Biorad TC10 automated cell counter and 1 x 10^6 cells/mL were resuspended in 100 µL of incubation buffer (PBS with 0.05% Bovine Serum Albumin). Mixed glial cultures were labeled using the mouse anti-GLAST-PE (20µg/mL) and mouse anti-CD11b –FITC (10µg/mL) at room temperature for 1 hr. Microglia cultures were incubated with CD11b-PE according to manufacturers instructions while astrocyte cultures were incubated with rabbit polyclonal anti-GLAST-488 (10µg/mL) at room temperature for 1 hr. After labeling, the cells were washed twice in incubation buffer and resuspended at a final volume of 500µL of PBS and stored at 37°C until analysis. Flow cytometry was performed on a Beckman Coulter CyAn $_{ADP}$ Flow Cytometry operating Summit software for data collection at Colorado State University's Flow Cytometry Core Facility. All further data analysis was done utilizing FlowJo software (version 10.1; FLOWJo, Ashland, OR).

Apoptosis Annexin V Live Cell Assay. Harvested cells from primary cultures of astrocytes, microglia, or mixed glia (astrocytes and microglia) were isolated from wildtype C57Bl/6 and astrocyte-specific IKK2 knock-out mice and treated with variable concentrations (0-100µM) of $MnCl_2$ for 8 hours. The cells were washed in cold phosphate- buffered saline (PBS) and then

suspended in annexin- binding buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$, pH 7.4). Followed by the addition of annexin V conjugate and Propidium Iodide to each sample. The samples were then incubated at room temperature for 15 minutes. After labeling, cells were resuspended at a final volume of 500µL of annexin binding buffer and analyzed immediately. Flow cytometry was performed on a Beckman Coulter CyAn $_{ADP}$ Flow Cytometry operating Summit software for data collection at Colorado State University's Flow Cytometry Core Facility. All further data analysis was done utilizing FlowJo software (version 10.1; FLOWJo, Ashland, OR).

RESULTS

Purity of Glia Cultures

Immunofluorescence and flow cytometry were used to assess the purity of microglia and astrocytes isolated from mixed glial cultures via a column free immune-magnetic method (Figure 19). Representative images of glial cultures is shown in Figure 19A, 19D and 19G. Quantitative counts for the percent total of cells labeled for IBA-1 and GFAP revealed that mixed glia cultures contained approximately 30% IBA-1 positive cells (microglia) and 68% astrocytes (Figure 19B). Microglia purification contained 97% IBA-1 positive stained cells (Figure 19E) and astrocyte purification cultures contained 91% astrocytes (71% GFAP +, 20% GFAP-; Figure 19H). Flow cytometry confirmed the glial cell populations using distinct cell surface markers for microglia (Cd11b) and astrocytes (GLAST/SLC1A3). The percentage of each glial cell type confirmed by flow cytometry in mixed glial cultures was ~ 21% Cd11b positive cells (microglia) and 61% GLAST positive cells (Figure 19C), microglia cultures were 96% Cd11b positive cells

(Figure 20F) and astrocyte cultures were 76% GLAST positive cells (Figure 20I), confirming similar distribution by immunofluorescence microscopy.

Conditional deletion of IKKβ in Astrocytes

To study the role of neuroinflammatory activation of astrocytes in the onset and progression of Parkinsons disease (PD), a mouse was generated with a conditional deletion of IKKβ, an essential kinase involved in the initiation of inflammation through the NFkβ pathway (Bonizzi et al., 2004). This was accomplished through breeding Ikkβ-floxed mice (Li et al., 2003) with hGFAP-Cre transgenic mice expressing Cre under the control of the human GFAP promoter (Zhuo et al., 2001). As shown in Fig 20, three generations of pairing were required to generate mice that had both the hGFAP-Cre allele and were homozygous for floxed-Ikkβ (hGFAP-Cre/Ikkβ$^{F/F}$) and thus for all experiments littermates homzygous for floxed-Ikkβ, but lacking hGFAP-Cre, known as Ikkβ$^{F/F}$, were used as controls. Confirmation of genotype for presence of hGFAP-Cre (Fig. 19A) and Ikkβ^{F} was achieved through PCR of ear tissue punches in adults and tail biopsies from neonatal mice.

To test the efficiency of Cre induced recombination in the brain, we cultured primary astrocytes from hGFAP-Cre/Ikkβ$^{F/F}$ and Ikkβ$^{F/F}$ mice and measured the deletion of Ikkβ at the genomic level using real time-PCR (data not shown). Cultured astrocytes from hGFAP-Cre/Ikkβ$^{F/F}$ neonates showed 71% deletion rate of Ikkβ (Fig. 20C) at the genomic level that corresponded to roughly a 70% loss of IKKβ protein measured by western blotting (Fig. 20c) and flow cytometry (Fig. 20B).

Column-Free immunomagnetic separation generates highly pure cultures of Astrocytes.

Mixed glia and astrocyte cultures were assessed for total glia composition via flow cytometry for Cd11b and GLAST (Figure 22a). Mixed glia cultures showed approximately 79% positive for GLAST and 12% positive for Cd11b. The data underwent compensation to take into account the spectral overlap of PE and FITC channel. Astrocyte purification was once again conducted and cells were subjected to flow cytometry to quantify their purification. The analysis revealed that approximately 97% of the cells stained positive for GLAST (Figure 22b), confirming a successful purification sequence.

Pharmacologic and genetic inhibition of NF-kB decreases glial-mediated neuronal cell death.

N2a cells were exposed to glia-conditioned media (GCM) and live cell flow cytometry was used to quantify the degree of apoptosis using annexin V and Propidium iodide co-stains. The data showed that $MnCl_2$-GCM is more potent inducer of neuronal cell death than other cell types. Additionally, IKK inhibition studies suggest that inflammatory signaling between microglia and astrocytes regulate neuronal injury.

DISSCUSSION AND CONCLUSIONS

The technology of flow cytometry has made a significant impact on many fields. There are few technologies that can evaluate so many parameters on small samples in short periods of time. The principle of evaluating each and every cell or particle that passes through the laser beam and to produce a highly-correlated data set is unique to flow cytometry. This work presents a practical application of flow cytometry to identify heterogeneous populations and validate microglia and astrocyte purification methods. The multi-parameter analyses

combined with an apoptosis study allowed us to robustly quantify desirable cellular properties under production conditions. Used in conjunction with other applications, these data provide a compelling contribution to scientific research.

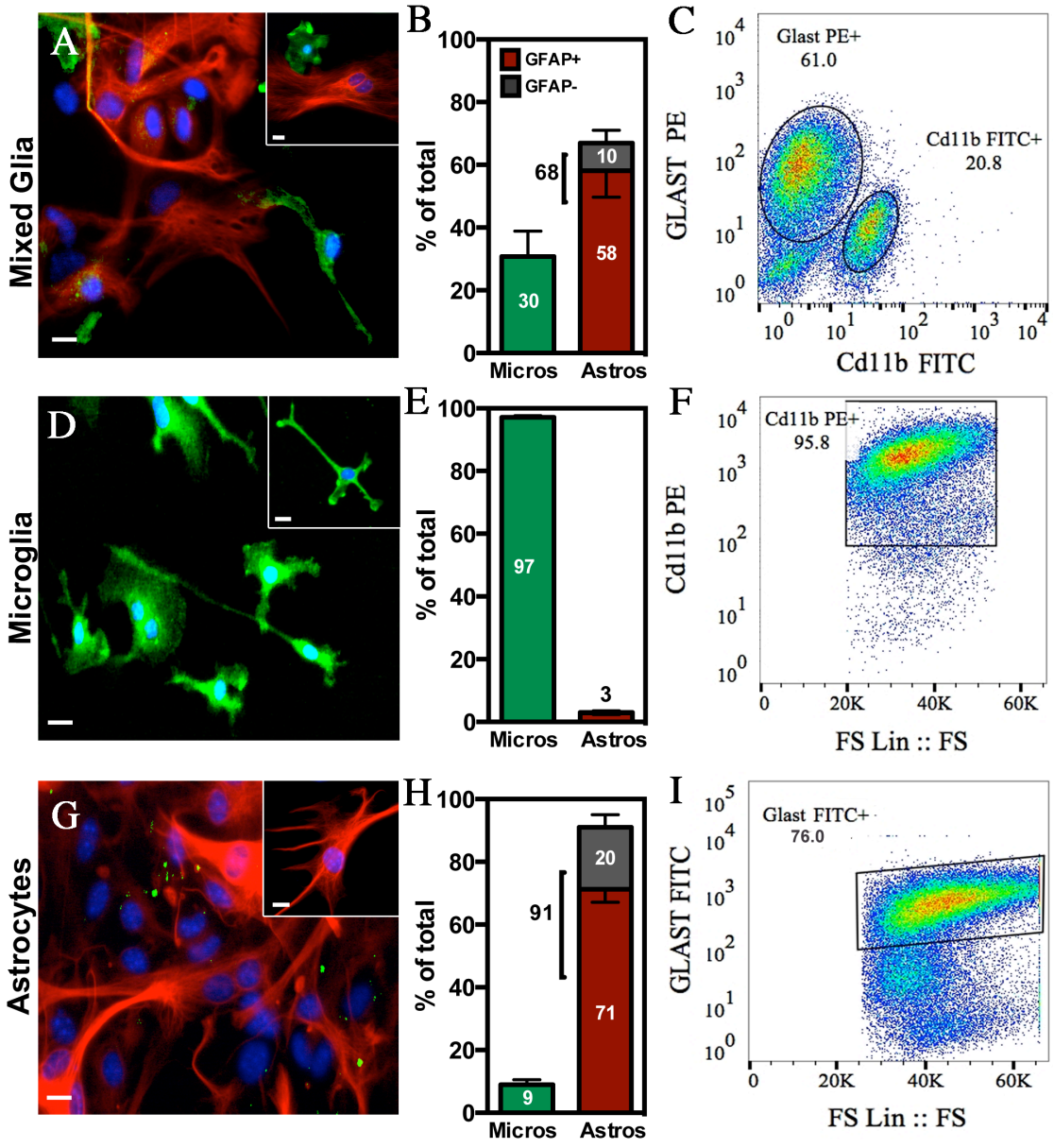

Figure 19. Column-Free immunomagnetic separation generates highly pure cultures of microglia. Mixed glia (A-C), microglial (D-F), and astrocyte (G-I) cultures were assessed for total glia composition via immunofluorescence for GFAP (red) and IBA-1 (green) positive cells or via flow cytometry for Cd11b and GLAST. Representative 20x images of mixed glia (A), microglial (B), and astrocyte (C) cultures with a 40x insert showing GFAP (red), IBA-1 (green), and DAPI (blue). Scale bars = 10 μm. Quantitative counts were determined for the number of glia present in mixed glia (B), microglia (E), and astrocyte (H) cultures by both positive immunoreactivity (colored) or by consistent morphology in the absence of positive staining (gray). Data are presented as mean percent of total cells per field ± SEM. Flow cytometry scatter plots showing the percentage of Cd11b or GLAST-positive cells for mixed glia (C), microglia (F), or astrocyte (I) cultures

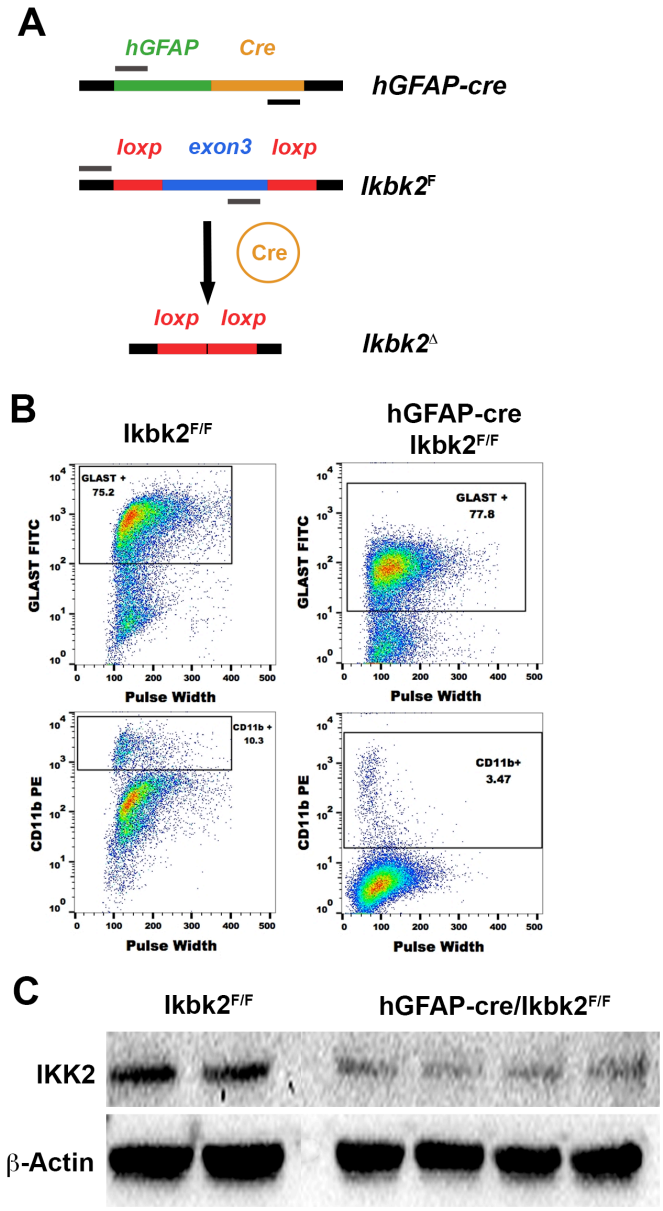

Figure 20. Conditional deletion of IKKβ in Astrocytes. (a) Three generations of pairing were required to generate mice that had both the hGFAP-Cre allele and were homozygous for floxed-Ikkβ (hGFAP-Cre/Ikkβ$^{F/F}$) and thus for all experiments littermates homzygous for floxed-Ikkβ, but lacking hGFAP-Cre, known as Ikkβ$^{F/F}$ (b and c) Cultured astrocytes from hGFAP-Cre/Ikkβ$^{F/F}$ were analyzed for protein expression by western blotting and flow cytometry.

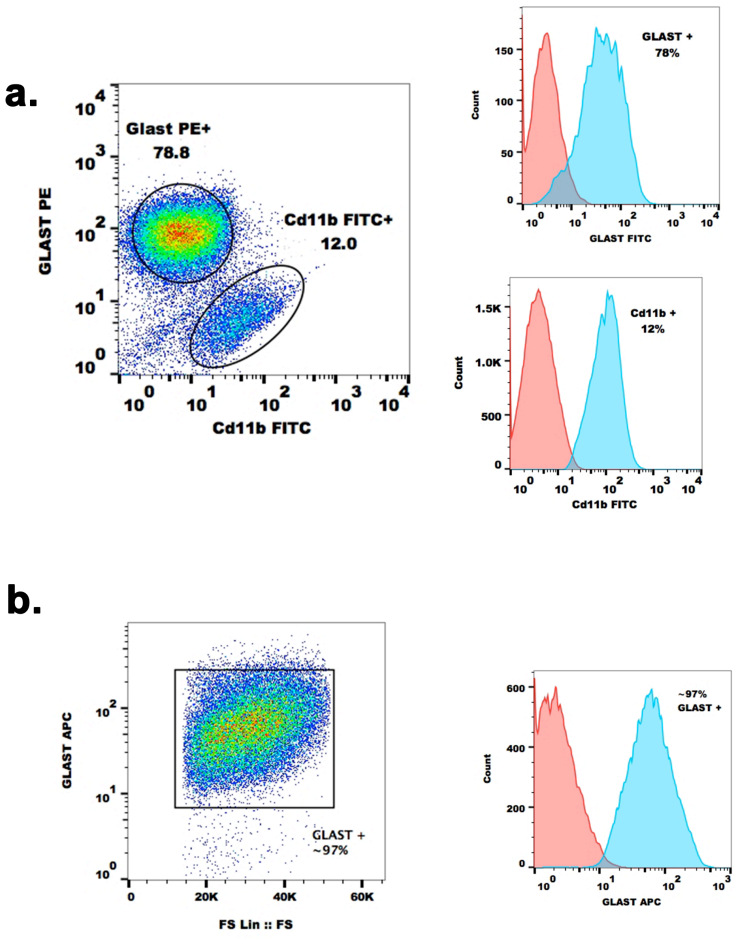

Figure 21. Column-Free immunomagnetic separation generates highly pure cultures of Astrocytes. Mixed glia (a) astrocyte (b) cultures were assessed for total glia composition via flow cytometry for Cd11b and GLAST. Flow cytometry scatter plots showing the percentage of Cd11b or GLAST-positive cells for mixed glia (a) astrocytes (b) cultures.

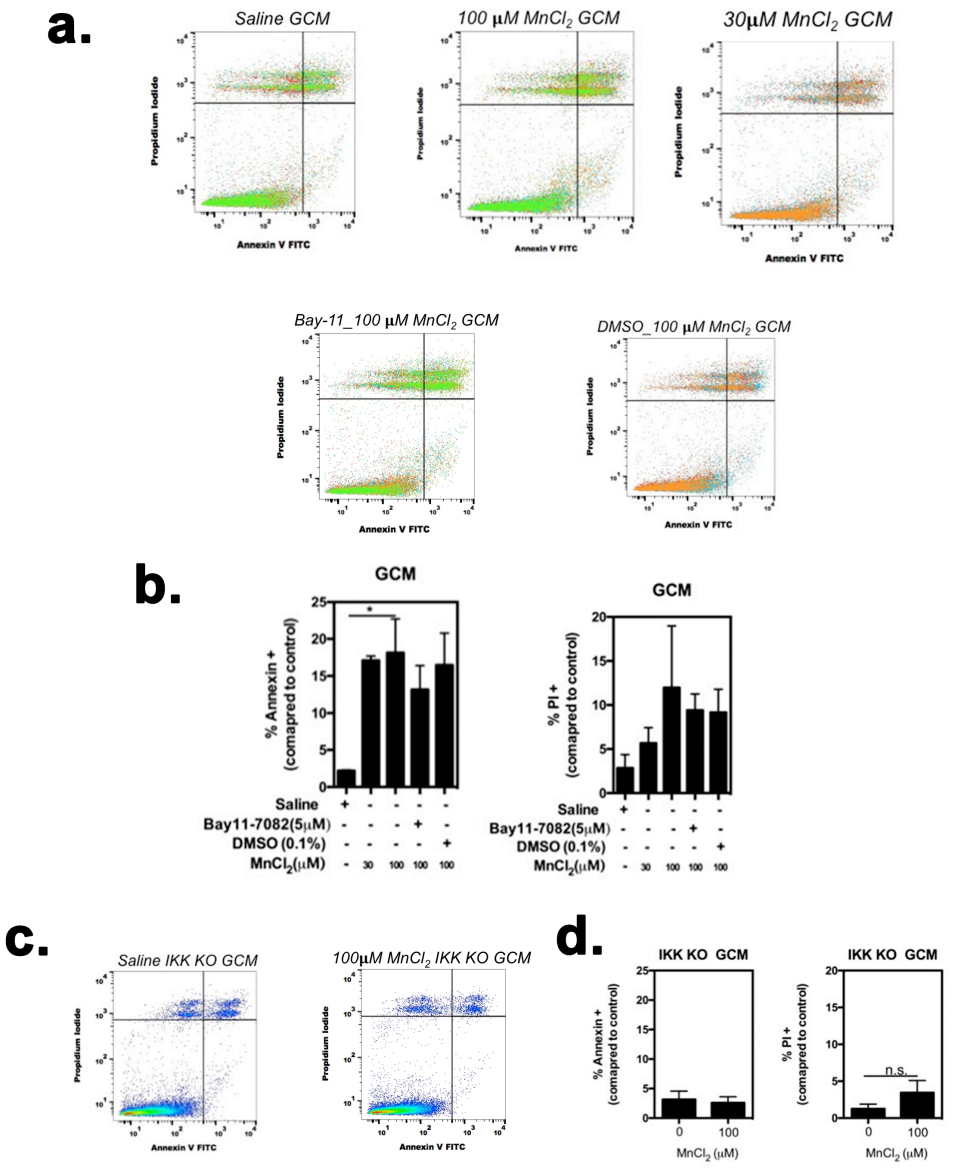

Figure 22. Pharmacologic and genetic inhibitions of NF-kB decrease glial-mediated neuronal cell death. (A) Graphs illustrate Annexin or PI positive N2A cell populations exposed to GCM. **(B)** Quantitative graphs show an increase in percent Annexin positive (left) in 100 **µ**M GCM treated cells and an increase in percent PI positive (right), while Bay11 treatment decreases both. **(C)** Graphs illustrate N2A cell populations, which are Annexin or PI positive. **(D)** Quantitative graphs show IKK KO to have less than GCM % Annexin (left) or % PI (right).

CHAPTER 6

DISCUSSIONS

Rheumatoid arthritis is a complex; multi-system disease with a primary site of inflammatory tissue damage is in the joint. Multiple cell types contribute to the pathogenic picture in rheumatoid synovium known as pannus, and studies implicate the T-cell as a key player of RA. Recent studies, provide increasing evidence that synovial fibroblasts cells also play a crucial role in both joint damage and propagation of inflammation (Kontoyiannis, D., et al., 2000). Current RA treatments alleviate joint pain, stiffness and slow the progression of joint damage. Effective treatment of RA has been hampered by the heterogeneity of the disease. The most common diagnosis when medical attention is sought out is defined as undifferential arthritis, indicating that arthritis is impossible to classify as a specific disease. Individuals with undifferential arthritis are followed over time and aren't formally diagnosed unless their symptoms persist for greater than six weeks and meet the current diagnostic criteria of RA (Lindstrom et al., 2010).

Once they meet the criteria, patients are administered therapies on a trial-and-error basis, with dose escalation and addition of other therapies, ultimately leading to a variation of treatments for a patient over the course of the disease. There are several therapeutic approaches for RA, disease-modifying anti-rheumatic drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs), and glucocorticoids. DMARDs target inflammation and by definition must reduce structural damage progression. NSAIDs reduce pain and stiffness while improving physical function, however they do not interfere with the pathogenesis of RA and therefore are not acceptable disease modifying treatments. On the other hand, glucocorticoids offer rapid

symptomatic and disease-modifying effects but are associated with serious long- term side effects (Smolen et al., 2016).

During the course of developing a new model of RA, we discovered that when RAW264.7 murine macrophages are stimulated with lipopolysaccharides (LPS), increases in proinflammatory markers including cyclooxygenase-2 (COX-2) and Inducible nitric oxide synthase (iNOS). With this knowledge we began treated induced RAW264.7 macrophage cells with a marketed non-steroidal anti-inflammatory drug as a positive control and subsequently treated with C-DIM12 that have been shown to have anti-inflammatory properties (Pathi et al., 2014). In the presence C-DIM12 there is statistical significance in the suppression of inflammatory response mediator COX-2, iNOS and NF-κB. The production of inflammatory mediators NO, PG, iNOS, and COX-2 are mainly regulated by NF-κB. Prostaglandins were significant to examine in addition to iNOS and COX-2 as these targets are relevant when investigating pain and inflammation. They aid in recovery at the sites of tissue damage and infection. These preliminary data show significant anti-inflammatory effects within novel C-DIM12. Moreover, it suggested if we could target specific effectors, cell-specific production and responses; such as prostaglandins, chemokine and cytokines provides a potential target for therapeutic intervention.

The utilization of primary synovial fibroblast for assessment of rheumatoid arthritis *in vitro* was an important tool necessary to provide a model that maintained normal cell morphology and many of the important markers and functions seen *in vivo*. C-DIMs have been shown to be involved in modulating inflammation and tumorigenicity, with the ability to modify selective inflammatory pathways in pancreatic, bladder, and colon cancer studies, as well as models of Parkinson's disease (De Miranda et al., 2013; De Miranda et al., 2014). The ultimate

goal was to develop cause directed, curative therapies, but this wasn't possible without better understanding of the cause or causes of rheumatoid arthritis.

Previous studies have observed that NR4A been demonstrated to be up-regulated in inflamed human synovial tissue, psoriatic skin, lung and colorectal cancer cells compared to normal tissue (McEvoy et al., 2002). In addition to the specific action of C-DIM12 was discovered to act on nuclear receptor Nurr1 transrepression of NF-κB on the NOS2 inflammatory promoter in microglia (De Miranda et al., 2015a). This led us to wonder if C-DIM12 may pharmacologically induce this pathway within synovial fibroblast. We observed that C-DIM12 reduced the expression of TNF-α induced adhesion molecules, pro-inflammatory mediators, IκBα and P65 NF-κB expression, NF-κB regulated cytokines in synovial fibroblasts in a Nurr1-dependent manner, losing its effect when Nurr1 was knocked down using dsRNAi. The transrepressive mechanism by which Nurr1 tethers nuclear corepressors (CoREST) to NF-κB-p65 elements, would provide insight on activation or co-activation by C-DIM12, however these experiments were out of the scope of this study.

FINAL CONCLUSIONS

It still remains a challenge to find small molecule therapies at reducing inflammatory components of RA, in which synovial fibroblasts continuously release pro-inflammatory cytokines leading to the destruction of the joint. The studies presented here, were designed to provide an understanding of the mechanism by which novel anti-inflammatory C-DIM12 compound provide anti-inflammatory benefits within synovial fibroblasts. The mechanisms by which C-DIM12 conveys anti-inflammatory properties were investigated. Analyses revealed a suppression of NF-κB induced synovial fibroblast. After exploring the specific action of C-DIM12 in synovial fibroblast, it suggests to act on nuclear receptor Nurr1 transrepression of NF-

κB in synovial fibroblasts. Taken together, these data presented provide a novel system capable of suppressing inflammation in an in-vitro RA model. This work provides insight into a new therapeutic agent targeting at the complex process of inflammation within the synovium.

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