ANGIOTENSIN II TYPE 2 RECEPTOR AGONIST PREVENTS THE PRO-INFLAMMATORY RESPONSE IN LPS TREATED HUMAN MACROPHAGES

By

ABDULKARIM ALSHAMMARI

(Under the Direction of Susan C. Fagan)

ABSTRACT

Stroke is a leading cause of long term disability and is associated with a 30% incidence of severe cognitive impairment. Sustained pro-inflammatory microglia activation contributes to, and our lab has shown that the Angiotensin II type 2 receptor (AT2R) agonist, compound 21 (C21) can prevent, the development of, PSCI. We hypothesized that activation of pro-inflammatory microglia and macrophages can be prevented with C21. This was assessed using a microglial cell line (C8-B4) and THP-1 derived macrophages. The reduction in the pro-inflammatory cell markers was assessed via RT-qPCR using the following genes, IL-1β, TNFα, and NOS2. Cells were either pre-treated, prior to LPS exposure, or post-treated after LPS treatment, with C21 (100 uM). C21 effectively reduced the expression of IL-1β in a concentration-dependent manner. Both pre- and post-treatment with C21 significantly reduced the expression of pro-inflammatory markers after LPS exposure in a mouse microglial cell line and human macrophages.

INDEX WORDS: Stroke; PSCI; Microglia; Macrophages; Neuroinflammation; Pro-inflammatory response; M1:M2; Compound 21; Angiotensin type 2 receptor (AT2R) agonist.

ANGIOTENSIN II TYPE 2 RECEPTOR AGONIST PREVENTS THE PRO-INFLAMMATORY RESPONSE IN LPS TREATED HUMAN MACROPHAGES

By

ABDULKARIM ALSHAMMARI

PHARM.D., Northern Border University, Saudi Arabia, 2015

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2020

ANGIOTENSIN II TYPE 2 RECEPTOR AGONIST PREVENTS THE PRO-INFLAMMATORY RESPONSE IN LPS TREATED HUMAN MACROPHAGES

By

ABDULKARIM ALSHAMMARI

Major Professor:	Susan C. Fagan
Committee:	Duo Zhang
	S. Priya Narayanan

Electronic Version Approved:

Ron Walcott
Interim Dean of the Graduate School
The University of Georgia
August 2020

DEDICATION

This thesis is dedicated to my parents

For their love, inspiration, and endless support.

I also dedicate this thesis to the loving memory of my brother, Rayan.

ACKNOWLEDGEMENTS

I would like to sincerely thank my advisor, Dr. Susan Fagan, for her valuable guidance and endless support throughout this master thesis. Her valuable comments and suggestions were very beneficial for the completion of this master thesis. I also, would like to thank my thesis committee members, Dr. Duo Zhang and Dr. Priya Narayanan, for their continuous help and support. Finally, I would like to express my heartfelt gratitude to all previous and current CET members for their encouragement, inspiration and support.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

CHAPTER 1

ANGIOTENSIN II TYPE 2 RECEPTOR AGONIST PREVENTS THE PRO-INFLAMMATORY RESPONSE IN LPS TREATED HUMAN MACROPHAGES

1) *Stroke incidence, complications, and treatment:*

a) *Stroke incidence, mortality, and statistics:*

Stroke is the fifth leading cause of death in the United States, indicted in one of every 20 deaths. The high incidence of stroke, coupled with improvements in stroke care, has led to a decrease in stroke mortality and an increase in stroke-induced disability. (Yang et al., 2017) (Lackland et al., 2014) Indeed, stroke was among the top 18 diseases contributing to years lived with disability in 2010. (Benjamin et al., 2018) Stroke survivors have an increased risk of developing a long-term disability, including sensorimotor and cognitive deficits. (Sun, Tan, & Yu, 2014)

b) *Post-Stroke Cognitive Impairment:*

Cognitive impairment frequently affects stroke survivors and is commonly referred to as post-stroke cognitive impairment (PSCI) under the umbrella pathology of vascular cognitive impairment. Globally, based on the variation in races, countries, and diagnostic criteria, the prevalence of post-stroke cognitive impairment ranges between 20% and 80%. (Sun et al., 2014). Generally, PSCI is defined as cognitive impairment persisting three to six months after stroke, but strict diagnostic criteria are lacking. PSCI causes extensive reductions in quality

of life and increases the burden of care, with many patients progressing to dementia (Mijajlovic et al., 2017). The Framingham study found 19.3 % of stroke patients developed dementia ten years after stroke. (Ivan et al., 2004) Stroke causes an abrupt and accelerated long term cognitive decline. Acutely after stroke, patients experience decreases in global perception, learning capability, and verbal memory, as well as a rapid decline in executive function and cognition. (Levine et al., 2015) Other factors, such as age and medical comorbidities like diabetes and hypertension, are likely to worsen and accelerate post-stroke cognitive impairment(Walker, Power, & Gottesman, 2017; Ward et al., 2018), (Srikanth, Quinn, Donnan, Saling, & Thrift, 2006) Hypertension impairs stroke recovery and increases PSCI risk through its effects on the cerebral vasculature (Iadecola & Gottesman, 2019). A prospective cohort of 796 patients with acute ischemic stroke revealed that abnormal blood pressure, either elevated or lowered, is associated with a higher risk of PSCI three months after stroke. (He et al., 2018) Despite the seriousness and frequency of PSCI, there are no FDA approved treatments for PSCI. Therefore, developing a therapy capable of reducing the risk of PSCI is crucial to post-stroke management.

c) *Approved Therapeutic Approach:*

Currently, reperfusion therapy is the only available option to treat patients after ischemic stroke (Powers et al., 2019). Reperfusion therapy can be achieved with thrombolytic drugs or mechanical thrombectomy. The primary purpose of reperfusion therapy is to restore cerebral blood flow to preserve non-infarcted ischemic brain tissues. Thrombolytic agents, such as tissue plasminogen

activator (tPA), are considered the gold standard of stroke treatment if initiated within 4.5 hours of stroke onset. (Lees et al., 2010) Reperfusion therapy should start as quickly as possible to protect the non-infarcted brain tissues and prevent further damage. The administration of tPA within 4.5 hours after acute ischemic stroke is associated with favorable clinical and functional outcomes. (Lees et al., 2016) Additional eligibility criteria of tPA further restrict its use to a small number of patients.

Only 15% to 32% of patients with ischemic stroke present within 3 hours of stroke onset, and of these, approximately 40% to 50% are eligible for tPA administration. (Messé et al., 2016) The safety and efficacy of endovascular treatment with mechanical thrombectomy were demonstrated in five randomized controlled trials, MR CLEAN, ESCAPE, SWIFT PRIME, REVASCAT, and EXTEND-IA. (Goyal et al., 2016) Based on the positive results from these trials, the number of stroke patients eligible for reperfusion therapy sharply increased. (Smith et al., 2017) Unlike tPA, mechanical thrombectomy has a more prolonged window of intervention beyond the 4.5 hours. In the DAWN and DEFUSE 3 trials, the effectiveness of mechanical thrombectomy beyond 6 hours was assessed in select patients suspected to have salvageable brain tissue. These two trials provided evidence that the use of endovascular treatment beyond 6 hours and up to 24 hours in select patients, is associated with less disability and better functional improvement 90 days after stroke. (Albers et al., 2018; Nogueira et al., 2018) Collectively, we can conclude that promptly starting reperfusion therapy for eligible patients is essential in stroke management. Reperfusion therapy has

long-term benefits by reducing disability and improving functional recovery after stroke. Despite the efficacy of reperfusion therapy, many stroke patients still develop significant disability due to ineligibility for both reperfusion therapies, and treatments capable of improving outcomes outside of the acute time window are needed.

2) *Inflammatory Response After Stroke:*

Over the past two decades, our knowledge of stroke pathophysiology has dramatically expanded, specifically to include the role of the immune system combined with a more advanced understanding of the neurovascular unit. The injury after ischemic stroke is mediated through a dynamic interaction between all the components of the neurovascular unit, which includes neuronal, glial, and vascular cells. (Thurgur & Pinteaux, 2019) Following the ischemic injury, the activation of glial cells leads to the release of cytokines, chemokines, and infiltration of peripheral leukocytes. Eventually, this furthers the inflammatory response and exacerbates the injury. (R. Jin, Yang, & Li, 2010)

Neuroinflammation is well known to be involved as a secondary injury following ischemic stroke and results in long-term consequences, such as PSCI. Following ischemic injury, damaged or dying neurons will release endogenous danger molecules known as Damage-associated molecular patterns (DAMPs). (Roh & Sohn, 2018) DAMPs release triggers the activation of glial cells, astrocytes and microglia, leading to changes in their morphology and function. Once activated, glial cells secrete various pro-inflammatory cytokines and chemokines. These cytokines, including interleukin-1β (IL- 1β) and tumor necrosis factor-alpha (TNFα), exacerbate

neuroinflammation. Furthermore, the release of pro-inflammatory cytokines will compromise the blood-brain barrier and lead to the infiltration of peripheral leukocytes. This infiltration leads to further damage in the subacute phase and contributes to secondary damage. (Amantea et al., 2015; Dabrowska, Andrzejewska, Lukomska, & Janowski, 2019)

Microglia, the resident innate immune cells, are key players in the inflammatory response in the central nervous system after stroke. (Lehnardt, 2010) Typically, in the brain, the primary role of resident microglia is to survey and maintain homeostasis. (Lenz & Nelson, 2018) In response to ischemic injury, microglia activate continually throughout the course of inflammation. Shortly after stroke, microglia are expected to adopt the anti-inflammatory or M2 phenotype. This healthier and anti-inflammatory phenotype results in enhanced phagocytosis, less inflammatory mediators, and improved neuronal survival. However, the M2 state is transient and the pro-inflammatory or M1 phenotype begins to dominate the injured area. The M1 microglia are characterized by less phagocytosis and higher production of the pro-inflammatory cytokines. (Stieler, Schumacher, Horst, & Fischer, 2012) The secretion of these pro-inflammatory cytokines initiate the inflammatory response, disrupt the blood-brain barrier, degrade the extracellular matrix, and trigger the infiltration of peripheral leukocytes into the central nervous system. (Rajkovic, Potjewyd, & Pinteaux, 2018) Altogether, these cellular processes will exacerbate the inflammatory response and worsen the injury.

Nevertheless, during the delayed phase of inflammation after stroke, microglia switch back to the M2 anti-inflammatory phenotype and facilitate functional

recovery. The anti-inflammatory microglia have favorable effects promoting blood-brain barrier repair, neurogenesis, and angiogenesis. These neuroprotective and anti-inflammatory effects are mediated via the release of different anti-inflammatory cytokines, such as IL-10, transforming growth factor (TGF)-β, IL-4, and IL-13. (Rajkovic et al., 2018) Accordingly, modulation of microglial inflammatory response after stroke toward an anti-inflammatory phenotype has emerged as one of the potential targets to reduce the long-term complications of stroke.

3) *Microglial Polarization as Therapeutic Target:*

   a) *Repurposing already approved drugs:*

Since the function of microglia/macrophages changes based on the activated phenotype, modulation of microglia/macrophage polarization toward more anti-inflammatory phenotype emerged as a potential therapeutic target. AMP-activated protein kinase (AMPK) is believed to counter regulate the inflammatory response in macrophages and shift its polarization toward the anti-inflammatory phenotype. (Sag, Carling, Stout, & Suttles, 2008) Metformin, a well-known and widely used antidiabetic drug, mediates its effect via the activation of AMPK. (Zhou et al., 2001) In an experimental stroke model, metformin shifted microglia/macrophage polarization toward an anti-inflammatory phenotype, and that led to favorable outcomes regarding functional recovery, promoting both neurogenesis and angiogenesis. (Q. Jin et al., 2014)

In addition to AMP-activated protein kinase (AMPK), other signaling pathways, such as notch signaling, are involved in the activation of microglia. Downregulation of the notch signaling pathway mediates the pro-inflammatory response and increases

pro-inflammatory markers such as TNF-α, IL-1β, and IL-6. Furthermore, the activation of the notch signaling pathway decreases the transcription of pro-inflammatory mediators such as NOS-II, TNF-α, IL-1β, and IL-6. (Grandbarbe et al., 2007) These findings confirm that the notch signaling pathway could be a potential target to modulate the polarization of microglia/macrophages and attenuate the inflammatory response. Statins, widely used lipid-lowering agents, showed beneficial effects regarding modulating the immune response and promoting anti-inflammatory effects. (Shimizu, Aikawa, Takayama, Libby, & Mitchell, 2003; Sorensen et al., 2011) Simvastatin is believed to mediate its anti-inflammatory response via the notch signaling pathway. In-vitro analysis showed that treating murine microglia with simvastatin inhibits M1 polarization and promotes an anti-inflammatory phenotype. Notch1 knockout in the microglia prevented this benefit suggesting that simvastatin may modulate the inflammatory response and shift the polarization of microglia toward M2 anti-inflammatory phenotype through the notch signaling pathway. (Wu et al., 2018) Collectively, these approved and widely used drugs were able to modulate the inflammatory response and promote anti-inflammatory effects via different signaling pathways.

b) Renin angiotensin system (RAS):

The brain renin-angiotensin system (RAS) is believed to be involved in the pathogenesis of stroke and other CNS diseases. Preclinical data showed that there is a direct correlation between angiotensin II (Ang II), the active neuropeptide in the renin-angiotensin system (RAS), and the severity of ischemic injury after stroke. (Walther et al., 2002) However, angiotensin II (Ang II) binds to different receptor

subtypes, mainly angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R). Angiotensin II type 1 receptor (AT1R) is known to be responsible for the pathological actions of angiotensin II (Ang II), while angiotensin II type 2 receptor (AT2R) opposes the action of AT1R. (de Gasparo, Catt, Inagami, Wright, & Unger, 2000) An experimental model of stroke shows that direct stimulation of AT2R via intracerebroventricular administration of CGP 42112, a peptide angiotensin II type 2 receptor agonist, was associated with a reduction in infarct volume, enhanced motor function, better neuronal survival, and increased microglial activation in the ischemic core. (McCarthy et al., 2012) Therefore, RAS modulation has emerged as a potential therapeutic target after stroke.

Studies from our lab were the first to demonstrate that compound 21 (C21), the first selective non-peptide angiotensin II type 2 receptor agonist, provides a neurovascular protective effect and enhances sustained functional improvement at 7 days after stroke. After a 3 hour middle cerebral artery occlusion (MCAO), a single dose of C21 was able to reduce the infarct size and enhance the behavioral outcome without affecting blood pressure. (Alhusban et al., 2015) Moreover, RAS modulation with either C21 or Candesartan, an angiotensin receptor blocker (ARB), showed favorable outcomes regarding PSCI (Ahmed et al., 2019). Post-stroke chronic administration of RAS modulators, C21 or Candesartan, prevented the development of PSCI in hypertensive rats. This effect was observed even with delayed administration of RAS modulators, 7 days after stroke. Along with preventing post-stroke cognitive decline, treatment with RAS modulators suppresses the sustained

microglial activation and prevents the microglial inflammatory response after stroke. (Ahmed et al., 2018)

Recently, we reported that delayed administration of C21 in diabetic rats, 3 days after stroke, improved sensorimotor function, cognitive deficit, and reduced mortality. These favorable outcomes were associated with a reduction in inflammatory response and demyelination, likely mediated through the modulation of microglial polarization (M1:M2 ratio). (Jackson et al., 2019) Thus, all these studies highlight the importance of brain RAS as it emerges as a potential therapeutic target for PSCI. Shifting the polarization of microglial cells toward the anti-inflammatory phenotype and reducing the pro-inflammatory response after stroke is believed to be one of the mechanisms in which C21 exerts its beneficial effects after stroke.

PROBLEM STATEMENT AND SPECIFIC AIMS

In our lab, we demonstrated that compound 21 (C21), the first selective non-peptide angiotensin II type 2 receptor agonist, enhanced neurovascular protection, improved functional outcomes, and prevented post-stroke cognitive impairment (PSCI). Along with these favorable outcomes, delayed administration of C21 reduced the pro-inflammatory response after MCAO in diabetic animals, suggesting that C21 mediates its beneficial effects through the modulation of microglial polarization. To further study the impact of C21 on microglial polarization, we assessed the direct effect of C21 on microglial mouse cell line (BV-2) and found a reduced inflammatory response and polarization shift toward the M2 phenotype with C21 treatment. Whether C21 causes this effect through direct stimulation of the AT2 receptor or an off-target effect remains unknown. Additionally, the findings in the mouse cell line may not effectively translate to humans. This study aims to

address these gaps in our knowledge. **Central Hypothesis:** Direct stimulation of angiotensin II type 2 receptor using C21 reduces the pro-inflammatory response in THP-1 differentiated human macrophages.

SPECIFIC AIMS

AIM 1: To determine the effect of C21 on pro-inflammatory markers in C8-B4, a mouse microglial cell line.

In this aim, we investigated the direct effect of C21 on M1 polarization markers, IL-1β, TNFα, and NOS2, using RT-PCR. To optimize the model, we conducted a concentration-dependent analysis to determine the most effective concentration of C21. Then, to confirm the involvement of AT2R, we used PD123,319, a selective non-peptide AT2 antagonist, to investigate whether it reversed C21 effects on microglial polarization.

AIM 2: To investigate whether C21 reduces the expression of pro-inflammatory markers in THP-1 differentiated human macrophages.

In this aim, we evaluated the direct effect of C21 on pro-inflammatory markers, IL-1β, and TNFα, using RT-PCR. Then, we used PD 123,319, a selective non-peptide AT2 antagonist, to determine whether the effects were mediated through the AT2R.

References:

Ahmed, H. A., Ishrat, T., Pillai, B., Bunting, K. M., Vazdarjanova, A., Waller, J. L., . . . Fagan, S. C. (2019). Angiotensin receptor (AT2R) agonist C21 prevents cognitive decline after permanent stroke in aged animals-A randomized double- blind pre-clinical study. *Behav Brain Res, 359*, 560-569. doi:10.1016/j.bbr.2018.10.010

Ahmed, H. A., Ishrat, T., Pillai, B., Fouda, A. Y., Sayed, M. A., Eldahshan, W., . . . Fagan, S. C. (2018). RAS modulation prevents progressive cognitive impairment after experimental stroke: a randomized, blinded preclinical trial. *J Neuroinflammation, 15*(1), 229. doi:10.1186/s12974-018-1262-x

Albers, G. W., Marks, M. P., Kemp, S., Christensen, S., Tsai, J. P., Ortega-Gutierrez, S., . . . Investigators, D. (2018). Thrombectomy for Stroke at 6 to 16 Hours with Selection by Perfusion Imaging. *N Engl J Med, 378*(8), 708-718. doi:10.1056/NEJMoa1713973

Alhusban, A., Fouda, A. Y., Bindu, P., Ishrat, T., Soliman, S., & Fagan, S. C. (2015). Compound 21 is pro-angiogenic in the brain and results in sustained recovery after ischemic stroke. *J Hypertens, 33*(1), 170-180. doi:10.1097/HJH.0000000000000364

Amantea, D., Micieli, G., Tassorelli, C., Cuartero, M. I., Ballesteros, I., Certo, M., . . . Bagetta, G. (2015). Rational modulation of the innate immune system for neuroprotection in ischemic stroke. *Frontiers in Neuroscience, 9*(147). doi:10.3389/fnins.2015.00147

Benjamin, E. J., Virani, S. S., Callaway, C. W., Chamberlain, A. M., Chang, A. R., Cheng, S., . . . Muntner, P. (2018). Heart Disease and Stroke Statistics—2018 Update: A Report From the American Heart Association. *Circulation, 137*(12), e67-e492. doi:doi:10.1161/CIR.0000000000000558

Dabrowska, S., Andrzejewska, A., Lukomska, B., & Janowski, M. (2019). Neuroinflammation as a target for treatment of stroke using mesenchymal stem cells and extracellular vesicles. *J Neuroinflammation, 16*(1), 178. doi:10.1186/s12974-019-1571-8

de Gasparo, M., Catt, K. J., Inagami, T., Wright, J. W., & Unger, T. (2000). International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacol Rev, 52*(3), 415-472. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/10977869

Goyal, M., Menon, B. K., van Zwam, W. H., Dippel, D. W. J., Mitchell, P. J., Demchuk, A. M., . . . Jovin, T. G. (2016). Endovascular thrombectomy after large-vessel ischaemic stroke: a meta-analysis of individual patient data from five randomised trials. *The Lancet, 387*(10029), 1723-1731. doi:https://doi.org/10.1016/S0140-6736(16)00163-X

Grandbarbe, L., Michelucci, A., Heurtaux, T., Hemmer, K., Morga, E., & Heuschling, P. (2007). Notch signaling modulates the activation of microglial cells. *Glia, 55*(15), 1519-1530. doi:10.1002/glia.20553

He, M., Wang, J. e., Liu, N., Xiao, X., Geng, S., Meng, P., . . . Wang, Y. (2018). Effects of Blood Pressure in the Early Phase of Ischemic Stroke and Stroke Subtype on Poststroke Cognitive Impairment. *Stroke, 49*(7), 1610-1617. doi:doi:10.1161/STROKEAHA.118.020827

Hu, X., Li, P., Guo, Y., Wang, H., Leak, R. K., Chen, S., . . . Chen, J. (2012). Microglia/Macrophage Polarization Dynamics Reveal Novel Mechanism of Injury Expansion After Focal Cerebral Ischemia. *Stroke, 43*(11), 3063-3070. doi:doi:10.1161/STROKEAHA.112.659656

Iadecola, C., & Gottesman, R. F. (2019). Neurovascular and Cognitive Dysfunction in Hypertension. *Circ Res, 124*(7), 1025-1044. doi:10.1161/CIRCRESAHA.118.313260

Ivan, C. S., Seshadri, S., Beiser, A., Au, R., Kase, C. S., Kelly-Hayes, M., & Wolf, P. A. (2004). Dementia After Stroke. *Stroke, 35*(6), 1264-1268. doi:doi:10.1161/01.STR.0000127810.92616.78

Jackson, L., Dong, G., Althomali, W., Sayed, M. A., Eldahshan, W., Baban, B., . . . Ergul, A. (2019). Delayed Administration of Angiotensin II Type 2 Receptor (AT2R) Agonist Compound 21 Prevents the Development of Post-stroke Cognitive Impairment in Diabetes Through the Modulation of Microglia Polarization. *Transl Stroke Res*. doi:10.1007/s12975-019-00752-5

Jin, Q., Cheng, J., Liu, Y., Wu, J., Wang, X., Wei, S., . . . Zhen, X. (2014). Improvement of functional recovery by chronic metformin treatment is associated with enhanced alternative activation of microglia/macrophages and increased angiogenesis and neurogenesis following experimental stroke. *Brain, Behavior, and Immunity, 40*, 131-142. doi:https://doi.org/10.1016/j.bbi.2014.03.003

Jin, R., Yang, G., & Li, G. (2010). Inflammatory mechanisms in ischemic stroke: role of inflammatory cells. *Journal of Leukocyte Biology, 87*(5), 779-789. doi:10.1189/jlb.1109766

Kim, E., & Cho, S. (2016). Microglia and Monocyte-Derived Macrophages in Stroke. *Neurotherapeutics, 13*(4), 702-718. doi:10.1007/s13311-016-0463-1

Lackland, D. T., Roccella, E. J., Deutsch, A. F., Fornage, M., George, M. G., Howard, G., . . . Translational, B. (2014). Factors influencing the decline in stroke mortality: a statement from the American Heart Association/American Stroke Association. *Stroke, 45*(1), 315-353. doi:10.1161/01.str.0000437068.30550.cf

Lees, K. R., Bluhmki, E., von Kummer, R., Brott, T. G., Toni, D., Grotta, J. C., . . . Hacke, W. (2010). Time to treatment with intravenous alteplase and outcome in stroke: an updated pooled analysis of ECASS, ATLANTIS, NINDS, and EPITHET trials. *The Lancet, 375*(9727), 1695-1703. doi:https://doi.org/10.1016/S0140-6736(10)60491-6

Lees, K. R., Emberson, J., Blackwell, L., Bluhmki, E., Davis, S. M., Donnan, G. A., . . . Zoppo, G. J. d. (2016). Effects of Alteplase for Acute Stroke on the Distribution of Functional Outcomes. *Stroke, 47*(9), 2373-2379. doi:doi:10.1161/STROKEAHA.116.013644

Lehnardt, S. (2010). Innate immunity and neuroinflammation in the CNS: The role of microglia in Toll-like receptor-mediated neuronal injury. *Glia, 58*(3), 253-263. doi:10.1002/glia.20928

Lenz, K. M., & Nelson, L. H. (2018). Microglia and Beyond: Innate Immune Cells As Regulators of Brain Development and Behavioral Function. *Frontiers in Immunology, 9*(698). doi:10.3389/fimmu.2018.00698

Levine, D. A., Galecki, A. T., Langa, K. M., Unverzagt, F. W., Kabeto, M. U., Giordani, B., & Wadley, V. G. (2015). Trajectory of Cognitive Decline After Incident Stroke. *JAMA, 314*(1), 41-51. doi:10.1001/jama.2015.6968

McCarthy, C. A., Vinh, A., Broughton, B. R., Sobey, C. G., Callaway, J. K., & Widdop, R. E. (2012). Angiotensin II type 2 receptor stimulation initiated after stroke causes neuroprotection in conscious rats. *Hypertension, 60*(6), 1531-1537. doi:10.1161/HYPERTENSIONAHA.112.199646

Messé, S. R., Khatri, P., Reeves, M. J., Smith, E. E., Saver, J. L., Bhatt, D. L., . . . Schwamm, L. H. (2016). Why are acute ischemic stroke patients not receiving IV tPA? Results from a national registry. *Neurology, 87*(15), 1565-1574. doi:10.1212/WNL.0000000000003198

Mijajlovic, M. D., Pavlovic, A., Brainin, M., Heiss, W. D., Quinn, T. J., Ihle-Hansen, H. B., . . . Bornstein, N. M. (2017). Post-stroke dementia - a comprehensive review. *BMC Med, 15*(1), 11. doi:10.1186/s12916-017-0779-7

Nogueira, R. G., Jadhav, A. P., Haussen, D. C., Bonafe, A., Budzik, R. F., Bhuva, P., . . . Investigators, D. T. (2018). Thrombectomy 6 to 24 Hours after Stroke with a Mismatch between Deficit and Infarct. *N Engl J Med, 378*(1), 11-21. doi:10.1056/NEJMoa1706442

Perego, C., Fumagalli, S., & De Simoni, M. G. (2011). Temporal pattern of expression and colocalization of microglia/macrophage phenotype markers following brain ischemic injury in mice. *J Neuroinflammation, 8*, 174. doi:10.1186/1742-2094-8-174

Powers, W. J., Rabinstein, A. A., Ackerson, T., Adeoye, O. M., Bambakidis, N. C., Becker, K., . . . Tirschwell, D. L. (2019). Guidelines for the Early Management of Patients With Acute Ischemic Stroke: 2019 Update to the 2018 Guidelines for the Early Management of Acute Ischemic Stroke: A Guideline for Healthcare Professionals From the American Heart Association/American Stroke Association. *Stroke, 50*(12), e344-e418. doi:10.1161/STR.0000000000000211

Rajkovic, O., Potjewyd, G., & Pinteaux, E. (2018). Regenerative Medicine Therapies for Targeting Neuroinflammation After Stroke. *Frontiers in Neurology, 9*(734). doi:10.3389/fneur.2018.00734

Roh, J. S., & Sohn, D. H. (2018). Damage-Associated Molecular Patterns in Inflammatory Diseases. *Immune network, 18*(4), e27-e27. doi:10.4110/in.2018.18.e27

Sag, D., Carling, D., Stout, R. D., & Suttles, J. (2008). Adenosine 5'-monophosphate-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype. *J Immunol, 181*(12), 8633-8641. doi:10.4049/jimmunol.181.12.8633

Shimizu, K., Aikawa, M., Takayama, K., Libby, P., & Mitchell, R. N. (2003). Direct anti-inflammatory mechanisms contribute to attenuation of experimental allograft arteriosclerosis by statins. *Circulation, 108*(17), 2113-2120. doi:10.1161/01.CIR.0000092949.67153.74

Smith, E. E., Saver, J. L., Cox, M., Liang, L., Matsouaka, R., Xian, Y., . . . Schwamm, L. H. (2017). Increase in Endovascular Therapy in Get With The Guidelines-Stroke After the Publication of Pivotal Trials. *Circulation, 136*(24), 2303-2310. doi:doi:10.1161/CIRCULATIONAHA.117.031097

Sorensen, P. S., Lycke, J., Eralinna, J. P., Edland, A., Wu, X., Frederiksen, J. L., . . . investigators, S. s. (2011). Simvastatin as add-on therapy to interferon beta-1a for relapsing-remitting multiple sclerosis (SIMCOMBIN study): a placebo-controlled randomised phase 4 trial. *Lancet Neurol, 10*(8), 691-701. doi:10.1016/S1474-4422(11)70144-2

Srikanth, V. K., Quinn, S. J., Donnan, G. A., Saling, M. M., & Thrift, A. G. (2006). Long-Term Cognitive Transitions, Rates of Cognitive Change, and Predictors of Incident Dementia in a Population-Based First-Ever Stroke Cohort. *Stroke, 37*(10), 2479-2483. doi:doi:10.1161/01.STR.0000239666.46828.d7

Stieler, K., Schumacher, U., Horst, A. K., & Fischer, N. (2012). XMRV induces cell migration, cytokine expression and tumor angiogenesis: are 22Rv1 cells a suitable

prostate cancer model? *PLoS One, 7*(7), e42321. doi:10.1371/journal.pone.0042321

Sun, J. H., Tan, L., & Yu, J. T. (2014). Post-stroke cognitive impairment: epidemiology, mechanisms and management. *Ann Transl Med, 2*(8), 80. doi:10.3978/j.issn.2305-5839.2014.08.05

Thurgur, H., & Pinteaux, E. (2019). Microglia in the Neurovascular Unit: Blood–Brain Barrier–microglia Interactions After Central Nervous System Disorders. *Neuroscience, 405*, 55-67. doi:https://doi.org/10.1016/j.neuroscience.2018.06.046

Walker, K. A., Power, M. C., & Gottesman, R. F. (2017). Defining the Relationship Between Hypertension, Cognitive Decline, and Dementia: a Review. *Curr Hypertens Rep, 19*(3), 24. doi:10.1007/s11906-017-0724-3

Walther, T., Olah, L., Harms, C., Maul, B., Bader, M., Hortnagl, H., . . . Mies, G. (2002). Ischemic injury in experimental stroke depends on angiotensin II. *FASEB J, 16*(2), 169-176. doi:10.1096/fj.01-0601com

Ward, R., Valenzuela, J. P., Li, W., Dong, G., Fagan, S. C., & Ergul, A. (2018). Poststroke cognitive impairment and hippocampal neurovascular remodeling: the impact of diabetes and sex. *Am J Physiol Heart Circ Physiol, 315*(5), H1402-H1413. doi:10.1152/ajpheart.00390.2018

Wattananit, S., Tornero, D., Graubardt, N., Memanishvili, T., Monni, E., Tatarishvili, J., . . . Kokaia, Z. (2016). Monocyte-Derived Macrophages Contribute to Spontaneous Long-Term Functional Recovery after Stroke in Mice. *J Neurosci, 36*(15), 4182-4195. doi:10.1523/JNEUROSCI.4317-15.2016

Wu, F., Luo, T., Mei, Y., Liu, H., Dong, J., Fang, Y., . . . Guo, Y. (2018). Simvastatin alters M1/M2 polarization of murine BV2 microglia via Notch signaling. *J Neuroimmunol, 316*, 56-64. doi:10.1016/j.jneuroim.2017.12.010

Yang, Q., Tong, X., Schieb, L., Vaughan, A., Gillespie, C., Wiltz, J. L., . . . George, M. G. (2017). Vital Signs: Recent Trends in Stroke Death Rates - United States, 2000-2015. *MMWR Morb Mortal Wkly Rep, 66*(35), 933-939. doi:10.15585/mmwr.mm6635e1

Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., . . . Moller, D. E. (2001). Role of AMP-activated protein kinase in mechanism of metformin action. *The Journal of Clinical Investigation, 108*(8), 1167-1174. doi:10.1172/JCI13505

CHAPTER 2

ANGIOTENSIN II TYPE 2 RECEPTOR AGONIST PREVENTS THE PRO-INFLAMMATORY RESPONSE IN LPS TREATED HUMAN MACROPHAGES[1]

[1] Alshammari, Abdulkarim et al. To be submitted to *Translational Stroke Research*

ABSTRACT

Stroke is a leading cause of long term disability and is associated with a 30% incidence of severe cognitive impairment. Sustained pro-inflammatory microglia activation contributes to, and our lab has shown that the Angiotensin II type 2 receptor (AT2R) agonist, compound 21 (C21), can prevent the development of, PSCI. We hypothesized that activation of pro-inflammatory microglia and macrophages can be prevented with C21. This was assessed using a microglial cell line (C8-B4) and THP-1 derived macrophages. The reduction in the pro-inflammatory cell markers was assessed via RT-qPCR using the following genes, IL-1β, TNFα, and NOS2. Cells were either pre-treated, prior to LPS exposure, or post-treated after LPS treatment, with C21 (100 uM). C21 effectively reduced the expression of IL-1β in a concentration-dependent manner. Both pre- and post-treatment with C21 significantly reduced the expression of pro-inflammatory markers after LPS exposure in a mouse microglial cell line and human macrophages.

INDEX WORDS: Stroke; PSCI; Microglia; Macrophages; Neuroinflammation; Pro-inflammatory response; M1:M2; Compound 21; Angiotensin type 2 receptor (AT2R) agonist.

1. Introduction:

In the United States, stroke is the 5th leading cause of death and a major cause of disability. (Yang et al., 2017) In 2010, stroke was among the top 25 diseases that contributed to years lived with disability. (Benjamin et al., 2018) Post-stroke cognitive impairment is an important contributor to long-term disability after stroke. A worldwide analysis showed that around 20% to 80% of stroke survivors develop post-stroke cognitive impairment and are likely to develop dementia. (Sun et al., 2014) Analysis from a cohort of the Framingham Study showed that stroke increases the risk of developing dementia; dementia was developed in 19.3% of the cases versus 11% of the control group. (Ivan et al., 2004)

Stroke is associated with both an acute decline in cognition and accelerated persistent cognitive decline. Data from a national cohort of participants aged 45 or older suggested that stroke causes accelerated and persistent declines in global cognition and executive function. (Levine et al., 2015) Additional risk factors, such as age and other comorbidities, are likely to accelerate the development of PSCI. An analysis of a population-based cohort of stroke patients found increasing age was associated with the diagnosis of new clinical dementia two years after stroke. (Srikanth et al., 2006) In addition, comorbidities, such as diabetes and hypertension, are associated with a higher risk of developing PSCI at 3 months after stroke. (He et al., 2018; Ward et al., 2018) Despite the seriousness of PSCI, there are no approved treatments that able to reduce the development of PSCI.

The ischemic injury following stroke is mediated through a dynamic interaction between all the components of the neurovascular unit. (Thurgur & Pinteaux, 2019)

Neuroinflammation is well known to contribute in stroke's long-term complications such as PSCI. After stroke, damaged neurons release dangerous endogenous molecules known as damage-associated molecular patterns, which trigger the activation of glial cells, astrocytes, and microglia. (Roh & Sohn, 2018) The activation of glial cells leads to secretion of pro-inflammatory cytokines such as, interleukin-1β (IL- 1β) and tumor necrosis factor-alpha (TNFα). The release of pro-inflammatory cytokines will exacerbate the injury, compromise the blood-brain barrier, and lead to the infiltration of peripheral leukocytes. Eventually, the infiltration of peripheral leukocytes through the blood-brain barrier will exacerbate neuroinflammation and contribute to secondary damage. (Amantea et al., 2015; Dabrowska et al., 2019)

Microglia, the resident innate immune cells, are key players in the inflammatory response in the central nervous system after stroke. (Lehnardt, 2010) Normally, the primary goal of resting microglia is to survey the brain to maintain homeostasis. (Lenz & Nelson, 2018) Following ischemic injury, microglia are activated into phenotypes throughout the course of inflammation. In the acute phase, microglia are often activated into the anti-inflammatory or M2 phenotype; a healthier and anti-inflammatory phenotype results in enhanced phagocytosis, and better neuronal survival. Shortly afterward, pro-inflammatory M1 phenotype microglia will begin dominating the injured area. (Stieler et al., 2012) The secreted pro-inflammatory cytokines will start the inflammatory response, disrupt the blood-brain barrier, degrade the extracellular matrix, and trigger the infiltration of peripheral leukocytes into the central nervous system. (Rajkovic et al., 2018) This cascade exacerbates the

inflammatory response and worsens the injury. However, in the delayed phase after stroke, microglia are expected to switch back to the anti-inflammatory phenotype (M2) and promote neurogenesis, angiogenesis, and functional recovery. The neuroprotective effects are mediated via the release of different anti-inflammatory cytokines such as, IL-10, transforming growth factor (TGF)-β, IL-4, and IL-13. (Rajkovic et al., 2018) Hence, modulation of microglial activation after stroke toward an anti-inflammatory phenotype has emerged as one of the potential targets to reduce PSCI.

Several studies have looked at the ability of already approved drugs to modulate the activation of microglia and macrophages toward an anti-inflammatory phenotype. Activation of the AMPK pathway is known to counteract the inflammatory response in macrophages/microglia. (Sag et al., 2008) Activation of AMPK pathway using Metformin, a well-known antidiabetic drug, shifts the polarization microglia/macrophages toward a more anti-inflammatory phenotype, which eventually led to favorable outcomes in angiogenesis, neurogenesis, and functional recovery. (Q. Jin et al., 2014; Zhou et al., 2001) Additionally, the downregulation of notch signaling pathway increases the pro-inflammatory response while its activation reduces the expression of pro-inflammatory mediators such as NOS-II, TNF-α, IL-1β, and IL-6. (Grandbarbe et al., 2007) Simvastatin, a widely used lipid-lowering agent, modulates the immune response and promotes anti-inflammatory effects. Statins' ability to shift the polarization of microglia/macrophages toward an anti-inflammatory phenotype was mediated through the activation of notch signaling pathway. (Shimizu et al., 2003; Sorensen et al., 2011; Wu et al., 2018) These data

indicate modulation of microglial polarization toward M2 phenotype can be achieved via diverse signaling pathways.

The brain RAS is involved in the pathogenesis of stroke and other CNS diseases. There is a direct correlation between angiotensin II (Ang II), the active neuropeptide in the RAS, and the severity of ischemic injury after stroke. (Walther et al., 2002) However, angiotensin II (Ang II) binds to different receptor subtypes, mainly angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R). AT1R is responsible for the pathological actions of Ang II, while AT2R opposes the action of AT1R. (de Gasparo et al., 2000)

Studies from our lab were the first to demonstrate that compound 21 (C21), the first selective non-peptide angiotensin II type 2 receptor agonist, has a neurovascular protective effect and enhances sustained functional improvement at 7 days after stroke. Following 3 hours MCAO, a single dose of C21 was able to reduce the infarct size and improve behavioral outcomes without affecting blood pressure. (Alhusban et al., 2015) Likewise, RAS modulation with either C21 or Candesartan, an angiotensin receptor blocker (ARB), showed favorable outcomes regarding PSCI. Post-stroke chronic administration of RAS modulators prevented the development of PSCI in hypertensive rats, suppressed sustained microglial activation, and prevented the microglial inflammatory response after stroke. (Ahmed et al., 2018)

Recently, we reported that delayed administration of C21 in diabetic rats, 3 days after stroke, improved both sensorimotor function and cognitive deficit and reduced mortality. These favorable outcomes were associated with a reduction in inflammatory response and demyelination, likely mediated through the modulation of microglial polarization

(M1:M2 ratio). (Jackson et al., 2019) This study aims to address whether C21 causes this effect through direct stimulation of the AT2 receptor or some off target effect and whether the findings in the mouse cell line would persist in humans. Since human microglia are inaccessible in vitro, we used a human monocyte cell line to model the immune response to stroke in patients. We anticipate that monocytes/macrophages will reflect the microglial response.

2. Materials and Methods

2.1. Cell Maintenance:

In this study, we used two different cell lines, a mouse microglial cell line (C8-B4) and a human monocyte cell line (THP-1). C8-B4 cells were cultured in DMEM media supplemented with 15% FBS and 1% Penicillin-Streptomycin at 37 °C in a humidified 5% CO2 incubator. For experiments, C8-B4 cells were seeded into 33 mm culture dishes at a density of 1 – 1.5 $\times 10^6$ cells/dish. After 24 hours, prior to any treatment, cells were washed with PBS and incubated overnight in serum free media. THP-1 cells were maintained in RPMI 1640 media supplemented with 10% FBS and 1% Streptomycin, Amphotericin B, and Penicillin at 37 °C in a humidified 5% CO2 incubator. For experiments, THP-1 cells were seeded into 33 mm culture dishes at a density of 1 – 1.5 $\times 10^6$ cells/dish. 5 ng/ml of Phorbol 12-myristate 13-acetate (PMA) was added to differentiate THP-1 monocyte into macrophages. After 24 hours incubation with PMA, cells are expected to be attached to the culture dish, indicating differentiated THP-1 macrophages.

Then, once cells' differentiation confirmed, cells were washed with PBS and incubated overnight in serum free media.

2.2. Treatment Conditions:

Prior to any intervention experiment, conditions were optimized to confirm the ability of C8-B4, and THP-1 derived macrophages to polarize into M1-like phenotype and express pro-inflammatory genes, IL-1β, TNFα, and NOS2 after treatment with LPS. Cells were treated with LPS (Lipopolysaccharide, 100 ng/ml) for 6 hours, and the expression of the pro-inflammatory markers was evaluated using RT-qPCR.

The direct effect of C21 on the polarization status of C8-B4 and THP-1 derived macrophages was determined by RT-qPCR. To find the optimal concentration of C21, we conducted a dose-response analysis assessed by the ability of different doses of C21, 1 uM, 10 uM, and 100 uM, to reduce the expression of IL-1β. Then, to evaluate whether C21 impacts the activation and polarization of microglia/macrophages by preventing (pre-treatment) or reversing (post-treatment) M1-like polarization, cells were either pre-treated, 30 min prior, or post-treated, 4 hours after LPS exposure, with C21 (100 uM). Finally, PD 123,319, a selective non-peptide AT2 antagonist, was used to determine if the C21 effects were mediated through AT2R agonism.

2.3. Quantitative Real Time RT-PCR

Quantitative gene expression analysis was done using SYBR green technology. Total RNA from treated C8-B4 and THP-1 derived macrophages was extracted using TRIzol™ Plus RNA Purification Kit. The concentration

and the purity of RNA were determined using NanoDrop spectrophotometer. Then, 0.5 ug of RNA was used to synthesize complementary DNA (cDNA) using high-capacity cDNA reverse transcription kit. Quantitative real-time PCR was performed using Sybr green master mix, TBP was used as a reference gene. Amplification condition was the following: initial pre-incubation at 95 °C for 10 min, followed by amplification of the target DNA for 40 cycles (95 °C for 15 seconds followed by 60 °C for 1 min). Primers sequences for the target genes, Il-1β - TNFα - NOS2, and the housekeeping gene, TBP, are as the following: *mouse TBP* forward 5′-TCAAACCCAGAATTGTTCTCC -3′, reverse 5′-GGGGTAGATGTTTTCAAATGC -3′; human *TBP* forward 5′-GATAAGAGAGCCACGAACCAC-3′, reverse 5′-CAAGAACTTAGCTGGAAAACCC-3′; *mouse IL-1β* forward 5′-TGGACCTTCCAGGATGAGGACA -3′, reverse 5′-GTTCATCTCGGAGCCTGTAGTG -3′; *human IL-1β* forward 5′-GGACAAGCTGAGGAAGATGC-3′, reverse 5′-TCGTTATCCCATGTGTCGAA -3′; *mouse TNFα* forward 5′-CCCTCACACTCAGATCATCTTCT-3′, reverse 5′-GCTACGACGTGGGCTACAG-3′; *human TNFα* forward 5′-AACCTCCTCTCTGCCATCAA-3′, reverse 5′-GGAAGACCCCTCCCAGATAG-3′; *mouse Nos2* forward 5′-GGCAGCCTGTGAGACCTTTG-3′, reverse 5′-GCATTGGAAGTGAAGCGTTTC-3′

2.4. Statistical Analysis:

Statistical analysis was performed using the GraphPad Prism software. Statistical analyses were done by analysis of variance (ANOVA) and a $P < 0.05$ was considered to be significantly different for two groups of data.

3. Results

3.1. Model Validation:

Prior to any intervention experiment, we validated the model regarding the ability of C8-B4 and THP-1 derived macrophages to polarize into M1-like phenotype and express the pro-inflammatory genes. After 6 hours LPS exposure, C8-B4 and THP-1 derived macrophages polarized into M1-like macrophages/microglia and expressed some pro-inflammatory markers. IL-1β and TNFα were expressed in both cell lines, while NOS2 was only detected in C8-B4. (Fig 1 A-C, Fig 2 A-B) Then, we performed a dose-response analysis to determine the optimal dose of C21. The effect of different doses of C21, 1 uM, 10 uM, and 100 uM, on the expression of IL-1β was used to determine the optimal dose for the next experiments. We found a dose-dependent reduction in the expression of IL-1β in response to C21 treatment; 100 uM of C21 effectively reduced the level of IL-1β to a level comparable to the control group. (Fig 3) Therefore, we decided to perform all the following experiments using 100 uM.

3.2. C21 Prevents the polarization of Microglia into M1-like phenotype:

The direct effect of C21 on the polarization status of a mouse microglial cell line, C8-B4, was assessed using RT-qPCR; the expression of both IL-1β and NOS2 was used to indicate the pro-inflammatory response. Briefly, C8-B4 cells

were seeded in 33 mm culture dishes at a density of $1 – 1.5 \times 10^6$ cells/dish. After 24 hours, cells were washed with PBS and incubated overnight in serum-free media. To evaluate the effect of C21 on preventing the polarization of microglia into M1-like phenotype, cells were pre-treated with C21 30 min prior LPS induction for 8 hours. Then, we looked at the expression of IL-1β and NOS2 to assess whether C21 prevents the expression of these pro-inflammatory markers. We found out that C21 efficiently prevents the polarization of microglia into the pro-inflammatory phenotype and expressed less IL-1β and NOS2. (Fig 4 A-B)

3.3. C21 Reverses the pro-inflammatory response in Microglia:

To ensure the therapeutic application of C21, its ability to reverse the expression of pro-inflammatory markers in microglia must be evaluated. To investigate this, C8-B4 cells were treated with C21 4 hours post LPS induction, and the expression of IL-1β and NOS2 was used to assess the inflammatory status. C21 effectively reversed the expression of IL--β and NOS2 toward less M1-like phenotype. (Fig 5 A-B) Collectively, we concluded that both pre- and post-LPS treatment with C21 blunted the expression of pro-inflammatory markers and ultimately modulated the inflammatory response induced by LPS.

3.4. *C21 Prevents the pro-inflammatory response in Human Macrophages:*

After we confirmed the ability of C21 to modulate the inflammatory response in the microglial mouse cell line, we wanted to ensure the translatability of our findings in human macrophages. To investigate the effect of C21 on the polarization status of THP-1 derived macrophages, THP-1 cells were seeded into 33 mm culture dishes at a density of $1 – 1.5 \times 10^6$ cells/dish. 5 ng/ml of PMA

was added to differentiate THP-1 monocyte into macrophages. After 24 hours of incubation with PMA, cell differentiation was confirmed, and cells were incubated overnight in serum-free media. To check the effect of C21 on the polarization status of THP-1 derived macrophages, cells were pre-treated with C21 30 min prior to LPS induction for 6 hours. Then, the expression of pro-inflammatory markers, IL-1β and TNFα, was assessed using RT-qPCR. As illustrated in Fig 6 A-B, C21 prevented the pro-inflammatory response and decreased the expression of IL-1β and TNFα.

3.5. ***C21 Modulates the Polarization of Human Macrophages by Reversing the pro-inflammatory response:***

As we showed earlier with microglia, to ensure the therapeutic applicability of C21, we wanted to check the capability of C21 to reverse the pro-inflammatory response in macrophages. Thus, THP-1 derived macrophages were treated with C21 4 hours post LPS induction. Then, the expression of IL-1β was assessed to indicate whether post-LPS treatment of C21 reversed the inflammatory response. As shown in Fig 7, post-LPS treatment with C21 effectively reversed the expression of IL-1β and reduced the pro-inflammatory response. Together, we concluded that both pre- and post-LPS treatment with C21 decreased the expression of pro-inflammatory markers and modulated the inflammatory response in human macrophages.

3.6. ***C21 Modulates the Polarization of Microglia/Macrophages via AT2R agonism:***

Whether C21 mediates its effects on microglia/macrophage polarization via AT2 receptor or another mechanism is unknown. To evaluate the involvement of AT2R in C21 mediated microglia/macrophages polarization, THP-1 derived macrophages were treated with different doses of C21, 1uM and 10 uM, with and without PD123,319 compound, a selective non-peptide AT2 antagonist. The expression of IL-1β was used to assess the ability of AT2 antagonist to reverse the reduction in pro-inflammatory response mediated by C21. Although there was no significant difference between the groups, we can see that the AT2 antagonist slightly prevents the reduction mediated by 1 uM of C21. (Fig 8) Yet, the involvement of AT2 antagonist in C21 mediated effects on microglia and macrophages need to be further investigated.

4. Discussion and Conclusion:

In the present study, we demonstrated that the direct stimulation of AT2R via C21 effectively modulated the activation and polarization of microglia/macrophages toward less pro-inflammatory phenotype. The main focus of the current study is to ensure that the effects of C21 on the polarization status of mouse microglial cell line would persist into human macrophages. Our findings indicated that C21 reduces the expression of pro-inflammatory markers in a dose-dependent manner in both mouse microglial cell line and THP-1 derived human macrophages. As expected, our data regarding the effect of C21 on the polarization status of microglia/macrophages were consistent in both human and mouse cell lines. To further emphasize the applicability

of our data, C21 modulated the inflammatory response even when used after LPS exposure, suggesting more therapeutic translatability.

After stroke, microglia follow a biphasic activation throughout the course of neuroinflammation. There will be a transient activation of the anti-inflammatory phenotype followed by the activation of M1-like phenotype. (Hu et al., 2012) However, during the delayed phase of neuroinflammation, microglia are designed to switch back to the anti-inflammatory phenotype to facilitate functional recovery, blood-brain barrier repair, neurogenesis, and angiogenesis. (Rajkovic et al., 2018)

Similarly, at the early stage after stroke, the newly recruited macrophages assume the anti-inflammatory phenotype and gradually changed to an M1-like phenotype. (Hu et al., 2012) (Perego, Fumagalli, & De Simoni, 2011) Recruitment of monocyte-derived macrophages into the ischemic brain contributes to the long-term spontaneous functional recovery. (Kim & Cho, 2016) Following MCAO, the depletion of circulating monocytes resulted in less monocyte-derived macrophages at the injury site and ultimately caused impaired recovery on sensorimotor function. Yet, monocyte-derived macrophages expressed high amounts of pro-inflammatory genes, IL-6, TNFα, IL-1β, and NOS2.(Wattananit et al., 2016) These findings illustrate the biphasic role of microglia/macrophages following stroke. Thus, modulating microglia/macrophage activation toward M2-like phenotype will be beneficial in reducing the inflammatory response and ultimately the long term functional recovery.

Our group was the first to report that C21 had neurovascular protective effects, causing sustained functional improvement at 7 days after stroke, reduced infarct size, and enhanced behavioral outcome. (Alhusban et al., 2015) Additionally, C21

exhibited favorable outcomes regarding PSCI, suppressing sustained microglial activation, and improving sensorimotor function and cognitive deficit. (Ahmed et al., 2018) (Ahmed et al., 2019). (Jackson et al., 2019) These favorable outcomes were associated with a reduction in the inflammatory response and are likely to be mediated through shifting the polarization of microglia/macrophages toward less pro-inflammatory phenotype.

Furthermore, the current study may provide an additional insight into how C21 exerts its beneficial effects after stroke. As we showed in this study, C21 has dual effects on the pro-inflammatory response, affecting both microglia and monocyte derived macrophages. Yet, we could not confirm that C21 effects on the polarization status was mediated through AT2 receptor. We need to further investigate the involvement of AT2 receptor and other pathways in mediating C21 effects on microglia/macrophages inflammatory response.

However, there are two major limitations in this study that could be addressed in future studies. First, we used a human monocyte cell line instead of primary human monocytes and cell lines do not fully reflect what is happening in vivo. Human primary cells will be more reflective in mimicking the physiological state of cells in-vivo. Second, the polarization status of microglia/macrophages was only assessed by gene expression using RT-qPCR. Flow cytometry would be better and give a more complete assessment of the inflammatory status of the cell markers. These two limitations should be considered in future studies.

To sum up, C21 modulates the polarization of microglia/macrophages toward less pro-inflammatory phenotype in a dose dependent manner. C21 effects were consistent in both mouse microglial cell line and human monocyte derived macrophages.

The relative expression of pro-inflammatory cytokines in C8-B4 cells

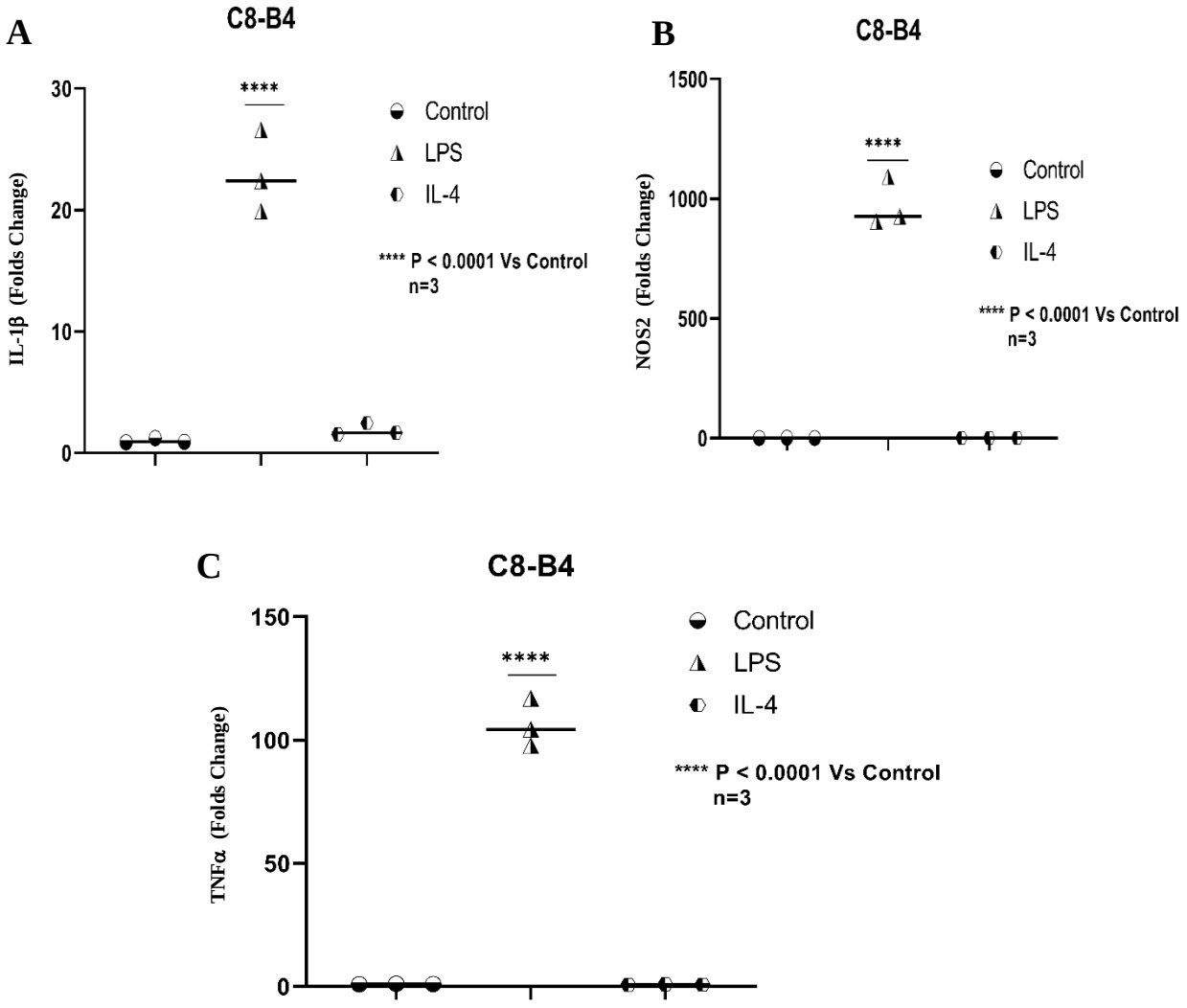

Figure 1:
C8-B4 cells were seeded at a density of 1 - 1.5 x 10^6 for 24 hours. Then, cells were incubated overnight with serum free media. The polarization of M1 and M2 phenotypes was induced by 6 hours incubation with either LPS (100ng/ml) or IL-4 (20ng/ml). (A): The relative expression of IL-1β in response to either LPS or IL-4 treatment. (B): The relative Expression of NOS2 in response to either LPS or IL-4 treatment. (C): The relative Expression of TNFα in response to either LPS or IL-4 treatment.

The relative expression of pro-inflammatory cytokines in THP-1 cells

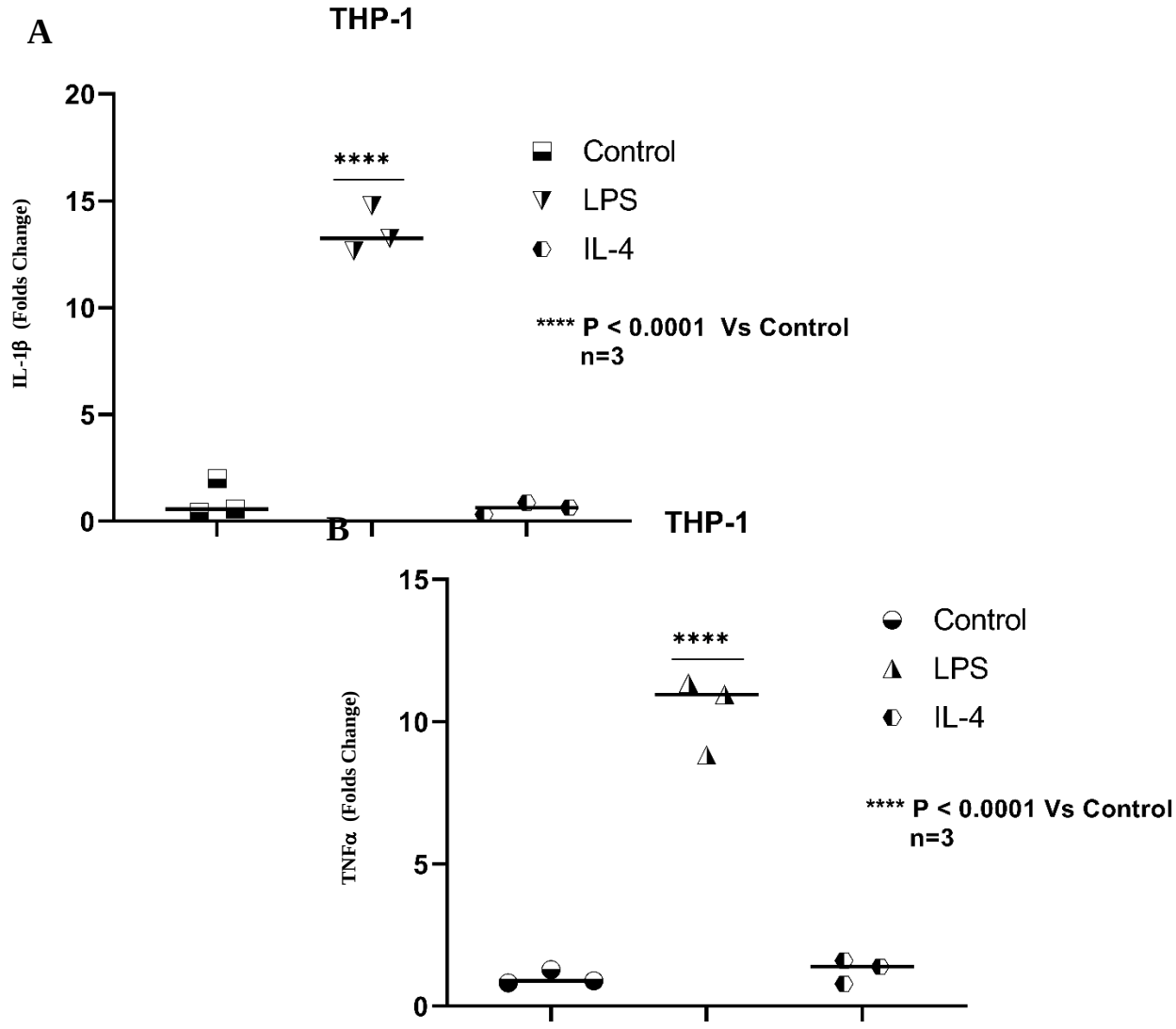

Figure 2:
THP-1 cells were seeded at a density of 1 - 1.5 x 106, cells were differentiated into Macrophages by 24 hours incubation with PMA (5 ng/ml). Then, cells were incubated overnight with serum free media. The polarization of M1 and M2 phenotypes was induced by 6 hours incubation with either LPS (100ng/ml) or IL-4 (20ng/ml). (A): The relative Expression of IL-1β in response to either LPS or IL-4 treatment. (B): The relative Expression of TNFα in response to either LPS or IL-4 treatment.

C21 reduces the expression of IL-β in a dose-dependent manner

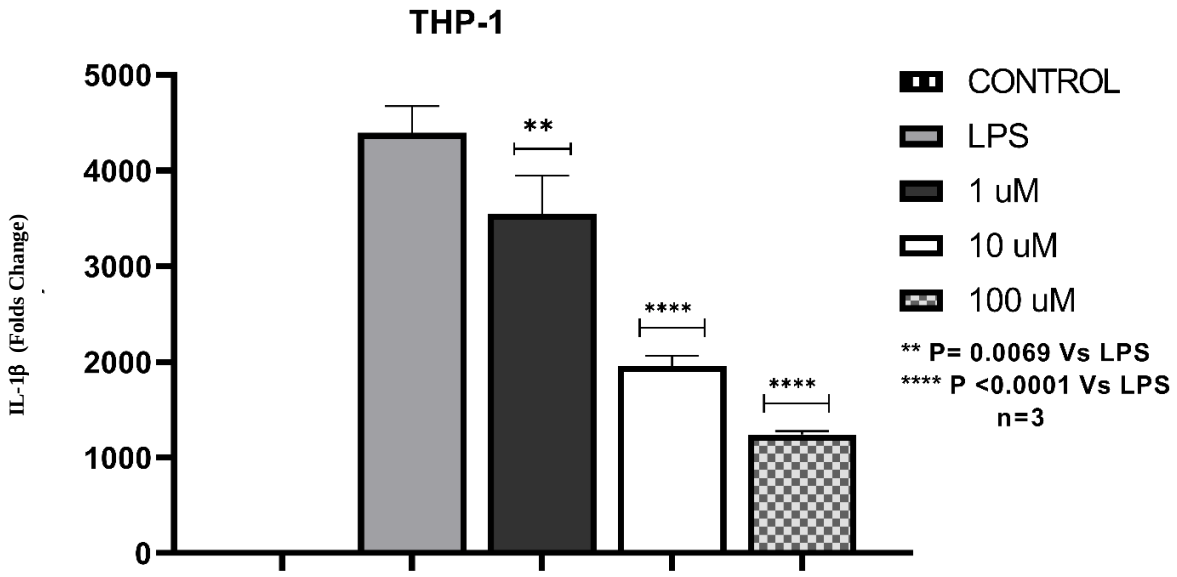

Figure 3:
THP-1 cells were seeded at a density of 1 - 1.5 x 106, cells were differentiated into Macrophages by 24 hours incubation with PMA (5 ng/ml). Then, cells were incubated overnight with serum free media. Cells were pre-treated with different concentration of C21 (1 uM, 10 uM, and 100 uM) 30 min prior LPS treatment, 100 ng/ml, for 6 hours. (A): The relative Expression of IL-1β in response to different doses of C21.

Compound 21 prevents the polarization of microglia into M1 phenotype

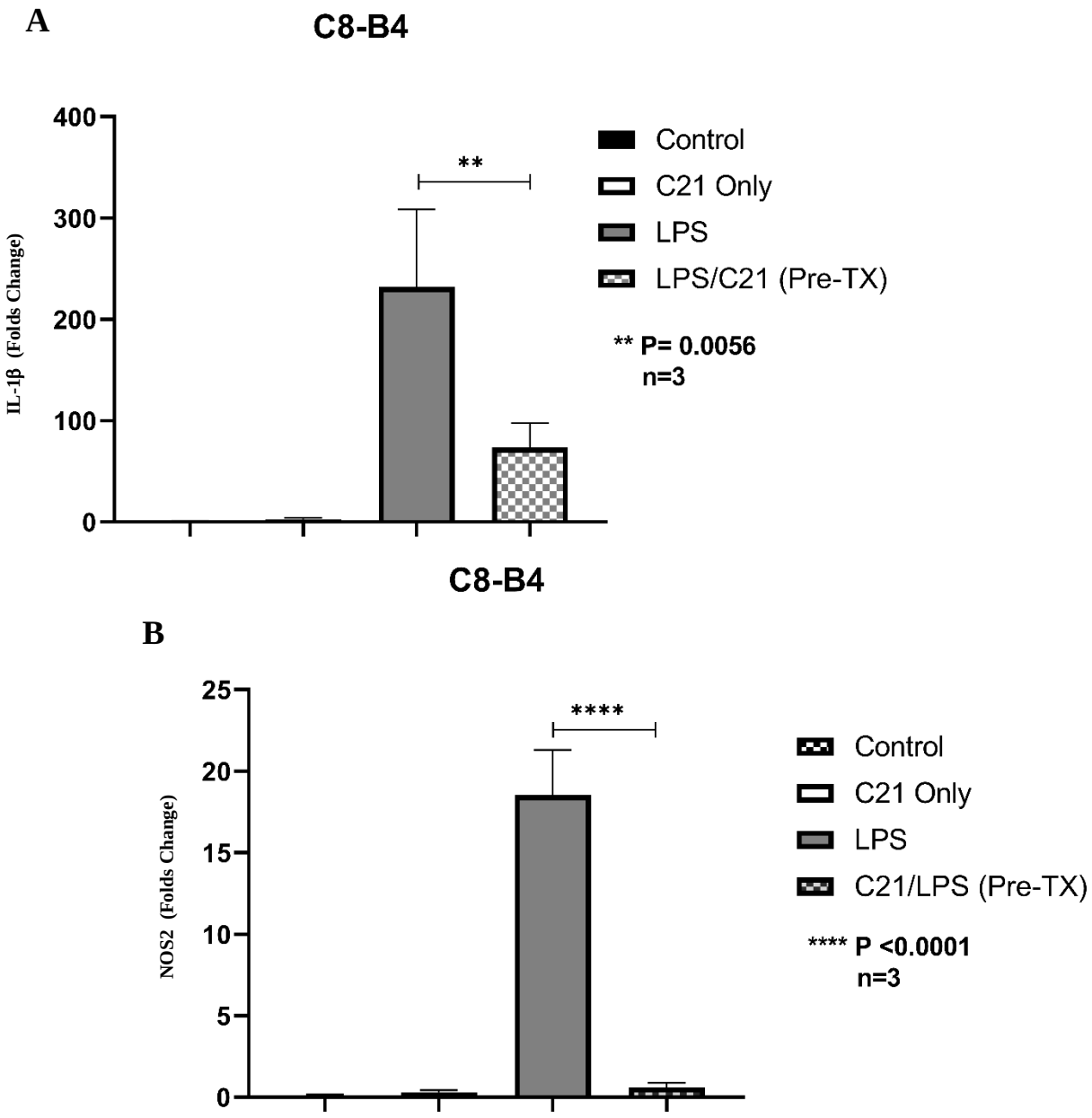

Figure 4:
C8-B4, mouse microglial cell line, Pre-treated with Compound 21 (100uM) 30 min prior LPS induction (100ng/ml) for 8 hours. C21 was able to prevent the expression of pro-inflammatory cytokines. (A): The relative Expression of IL-1β in response to C21/LPS treatment. (B): The relative Expression of NOS2 in response to C21/LPS treatment.

Compound 21 reverses the pro-inflammatory activation of Microglia

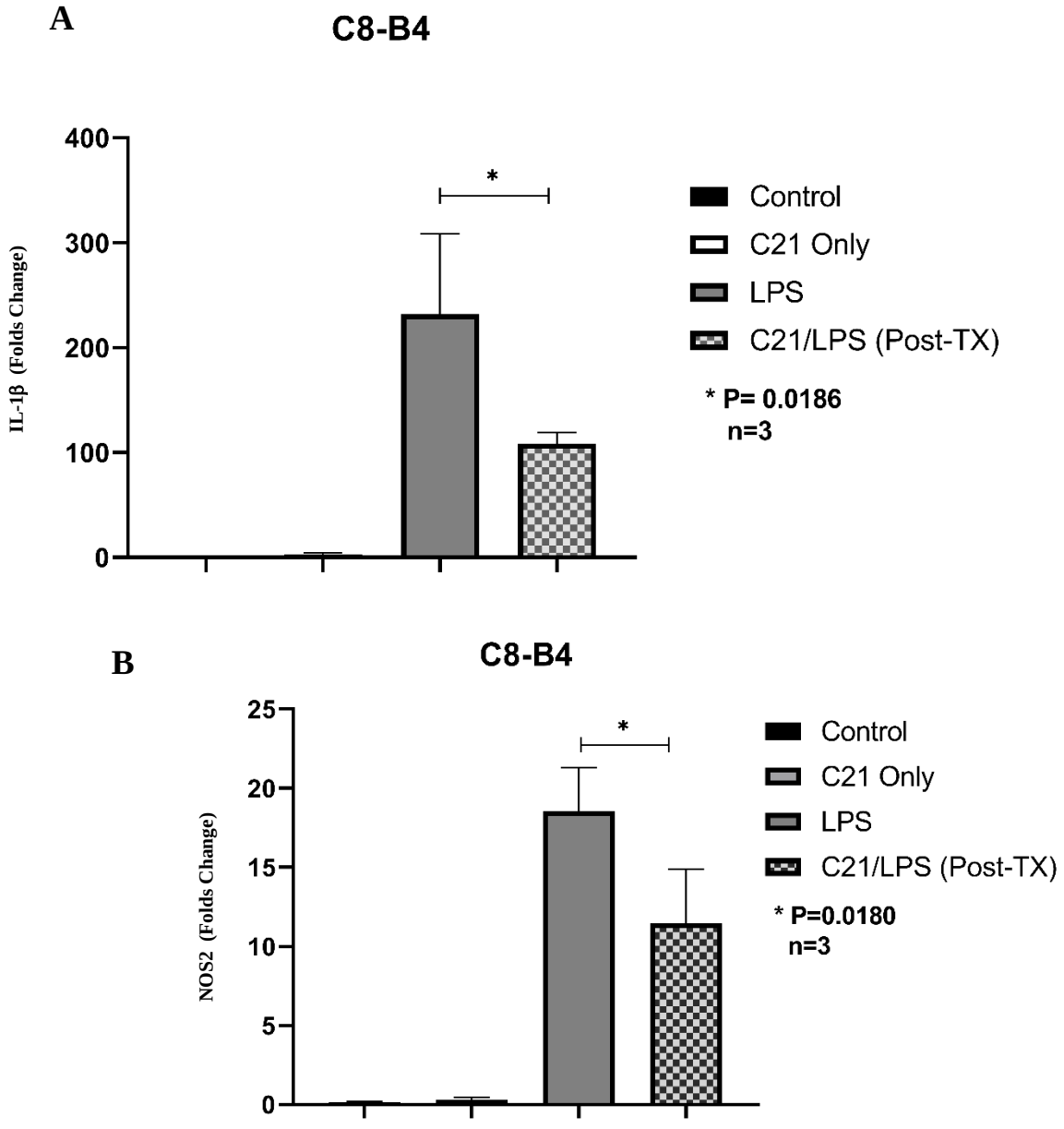

Figure 5:
C8-B4, mouse microglial cell line, treated with LPS (100ng/ml) for 4 hours and then incubated with C21 (100uM) for additional 4 hours. C21 was able to reverse the expression of pro-inflammatory cytokines. (A): The relative Expression of IL-1β in response to C21/LPS treatment. (B): The relative Expression of NOS2 in response to C21/LPS treatment.

Compound 21 prevents the polarization of Macrophages into M1 phenotype

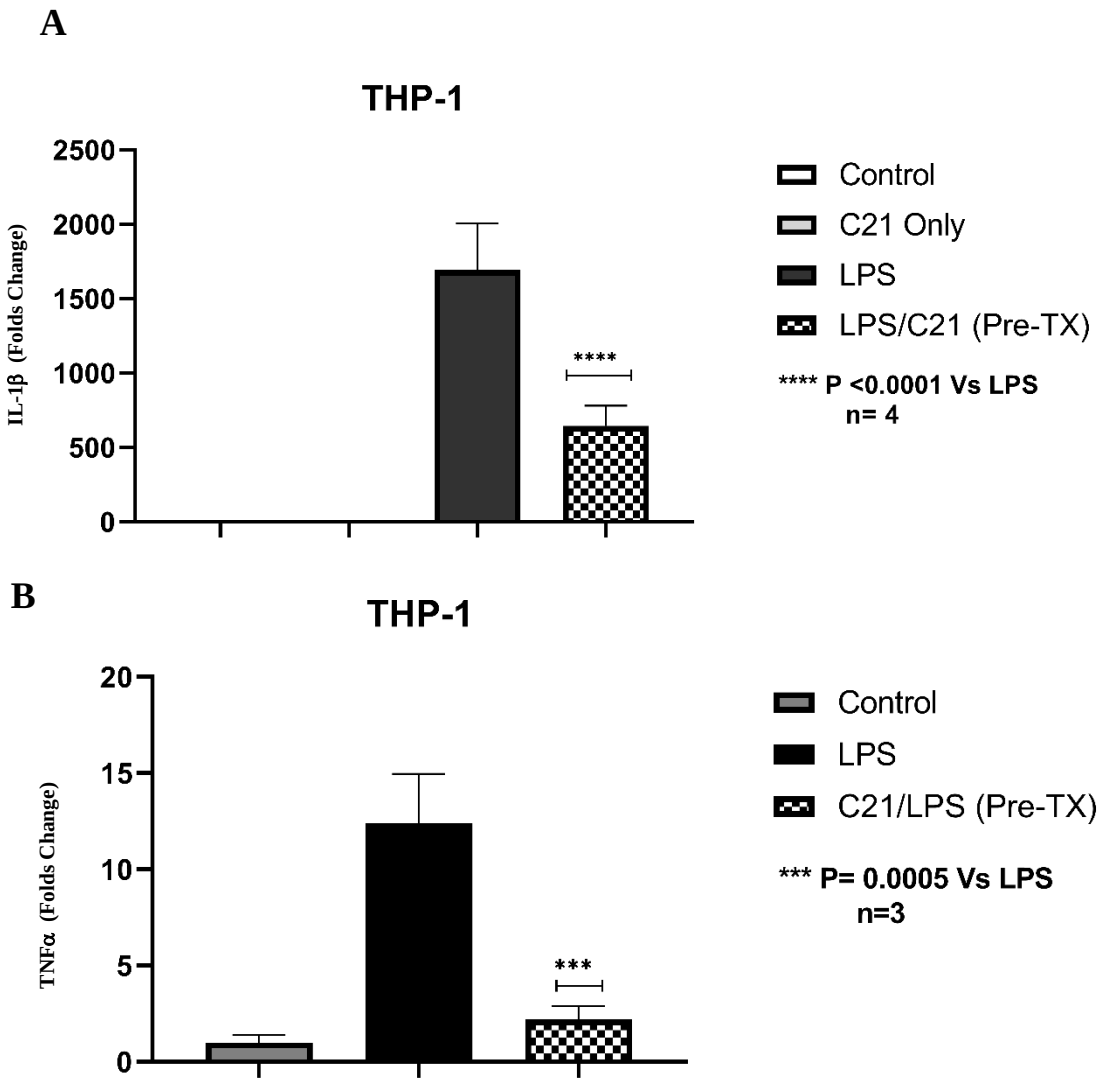

Figure 6:
THP-1 derived macrophages were pre-treated with C21 (100uM) 30 min prior LPS treatment for 6 hours. C21 was able to reverse the expression of pro-inflammatory cytokines. (A): The relative Expression of IL-1β in response to C21/LPS treatment. (B): The relative Expression of TNFα in response to C21/LPS treatment.

Compound 21 reverses the polarization of Macrophages into M1 phenotype

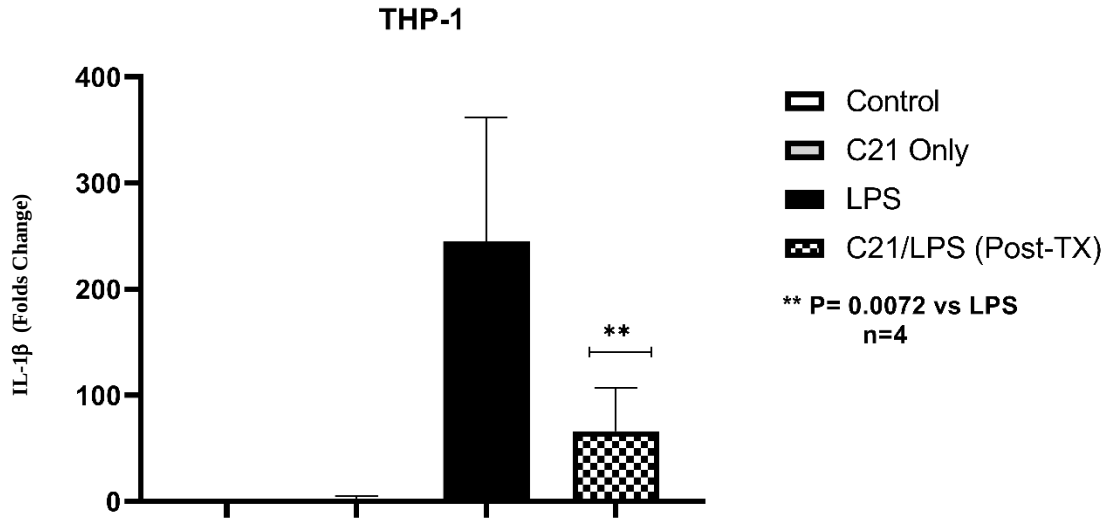

Figure 7:
THP-1 derived macrophages were post-treated with C21 (100uM) 4 hours after LPS treatment for 8 hours. C21 was able to reverse the expression of pro-inflammatory cytokines. (A): The relative Expression of IL-1β in response to C21/LPS treatment.

Compound 21 effects on the polarization status of Microglia/Macrophages was not mediated through AT2R agonism

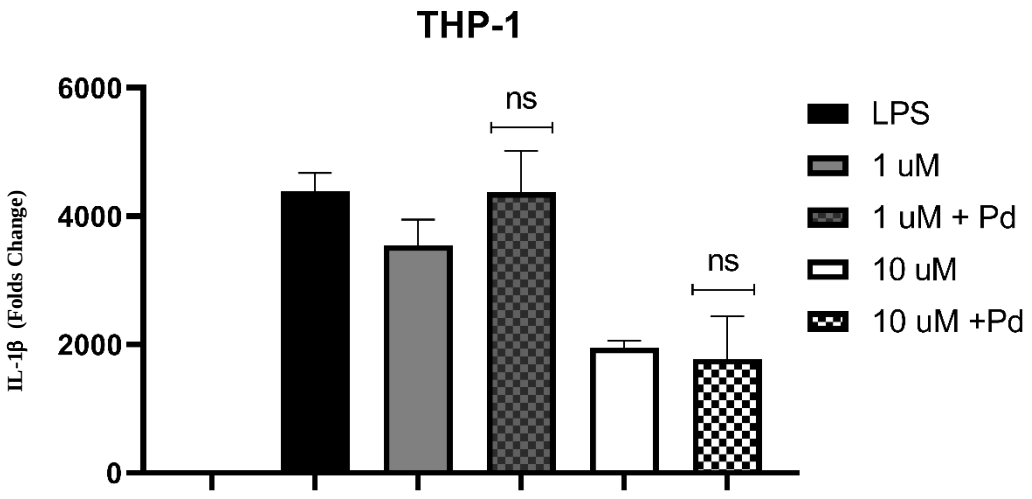

Figure 8:
THP-1 differentiated macrophages were treated with different doses of C21 (1 uM and 10 uM) and PD 123,319 (10 uM), a selective non-peptide AT2 antagonist, to evaluate whether C21 effects on the activation and polarization of microglia/macrophages is mediated through AT2R agonism. (A): The relative expression of IL-1B in response to different doses of C21 and PD123,319 compound. PD123,319 compound did not reverse the expression IL-1B

References:

Ahmed, H. A., Ishrat, T., Pillai, B., Bunting, K. M., Vazdarjanova, A., Waller, J. L., . . . Fagan, S. C. (2019). Angiotensin receptor (AT2R) agonist C21 prevents cognitive decline after permanent stroke in aged animals-A randomized double- blind pre-clinical study. *Behav Brain Res, 359*, 560-569. doi:10.1016/j.bbr.2018.10.010

Ahmed, H. A., Ishrat, T., Pillai, B., Fouda, A. Y., Sayed, M. A., Eldahshan, W., . . . Fagan, S. C. (2018). RAS modulation prevents progressive cognitive impairment after experimental stroke: a randomized, blinded preclinical trial. *J Neuroinflammation, 15*(1), 229. doi:10.1186/s12974-018-1262-x

Albers, G. W., Marks, M. P., Kemp, S., Christensen, S., Tsai, J. P., Ortega-Gutierrez, S., . . . Investigators, D. (2018). Thrombectomy for Stroke at 6 to 16 Hours with Selection by Perfusion Imaging. *N Engl J Med, 378*(8), 708-718. doi:10.1056/NEJMoa1713973

Alhusban, A., Fouda, A. Y., Bindu, P., Ishrat, T., Soliman, S., & Fagan, S. C. (2015). Compound 21 is pro-angiogenic in the brain and results in sustained recovery after ischemic stroke. *J Hypertens, 33*(1), 170-180. doi:10.1097/HJH.0000000000000364

Amantea, D., Micieli, G., Tassorelli, C., Cuartero, M. I., Ballesteros, I., Certo, M., . . . Bagetta, G. (2015). Rational modulation of the innate immune system for neuroprotection in ischemic stroke. *Frontiers in Neuroscience, 9*(147). doi:10.3389/fnins.2015.00147

Benjamin, E. J., Virani, S. S., Callaway, C. W., Chamberlain, A. M., Chang, A. R., Cheng, S., . . . Muntner, P. (2018). Heart Disease and Stroke Statistics—2018 Update: A Report From the American Heart Association. *Circulation, 137*(12), e67-e492. doi:doi:10.1161/CIR.0000000000000558

Dabrowska, S., Andrzejewska, A., Lukomska, B., & Janowski, M. (2019). Neuroinflammation as a target for treatment of stroke using mesenchymal stem cells and extracellular vesicles. *J Neuroinflammation, 16*(1), 178. doi:10.1186/s12974-019-1571-8

de Gasparo, M., Catt, K. J., Inagami, T., Wright, J. W., & Unger, T. (2000). International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacol Rev, 52*(3), 415-472. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/10977869

Goyal, M., Menon, B. K., van Zwam, W. H., Dippel, D. W. J., Mitchell, P. J., Demchuk, A. M., . . . Jovin, T. G. (2016). Endovascular thrombectomy after large-vessel ischaemic stroke: a meta-analysis of individual patient data from five randomised trials. *The Lancet, 387*(10029), 1723-1731. doi:https://doi.org/10.1016/S0140-6736(16)00163-X

Grandbarbe, L., Michelucci, A., Heurtaux, T., Hemmer, K., Morga, E., & Heuschling, P. (2007). Notch signaling modulates the activation of microglial cells. *Glia, 55*(15), 1519-1530. doi:10.1002/glia.20553

He, M., Wang, J. e., Liu, N., Xiao, X., Geng, S., Meng, P., . . . Wang, Y. (2018). Effects of Blood Pressure in the Early Phase of Ischemic Stroke and Stroke Subtype on Poststroke Cognitive Impairment. *Stroke, 49*(7), 1610-1617. doi:doi:10.1161/STROKEAHA.118.020827

Hu, X., Li, P., Guo, Y., Wang, H., Leak, R. K., Chen, S., . . . Chen, J. (2012). Microglia/Macrophage Polarization Dynamics Reveal Novel Mechanism of Injury Expansion After Focal Cerebral Ischemia. *Stroke, 43*(11), 3063-3070. doi:doi:10.1161/STROKEAHA.112.659656

Iadecola, C., & Gottesman, R. F. (2019). Neurovascular and Cognitive Dysfunction in Hypertension. *Circ Res, 124*(7), 1025-1044. doi:10.1161/CIRCRESAHA.118.313260

Ivan, C. S., Seshadri, S., Beiser, A., Au, R., Kase, C. S., Kelly-Hayes, M., & Wolf, P. A. (2004). Dementia After Stroke. *Stroke, 35*(6), 1264-1268. doi:doi:10.1161/01.STR.0000127810.92616.78

Jackson, L., Dong, G., Althomali, W., Sayed, M. A., Eldahshan, W., Baban, B., . . . Ergul, A. (2019). Delayed Administration of Angiotensin II Type 2 Receptor (AT2R) Agonist Compound 21 Prevents the Development of Post-stroke Cognitive Impairment in Diabetes Through the Modulation of Microglia Polarization. *Transl Stroke Res*. doi:10.1007/s12975-019-00752-5

Jin, Q., Cheng, J., Liu, Y., Wu, J., Wang, X., Wei, S., . . . Zhen, X. (2014). Improvement of functional recovery by chronic metformin treatment is associated with enhanced alternative activation of microglia/macrophages and increased angiogenesis and neurogenesis following experimental stroke. *Brain, Behavior, and Immunity, 40*, 131-142. doi:https://doi.org/10.1016/j.bbi.2014.03.003

Jin, R., Yang, G., & Li, G. (2010). Inflammatory mechanisms in ischemic stroke: role of inflammatory cells. *Journal of Leukocyte Biology, 87*(5), 779-789. doi:10.1189/jlb.1109766

Kim, E., & Cho, S. (2016). Microglia and Monocyte-Derived Macrophages in Stroke. *Neurotherapeutics, 13*(4), 702-718. doi:10.1007/s13311-016-0463-1

Lackland, D. T., Roccella, E. J., Deutsch, A. F., Fornage, M., George, M. G., Howard, G., . . . Translational, B. (2014). Factors influencing the decline in stroke mortality: a statement from the American Heart Association/American Stroke Association. *Stroke, 45*(1), 315-353. doi:10.1161/01.str.0000437068.30550.cf

Lees, K. R., Bluhmki, E., von Kummer, R., Brott, T. G., Toni, D., Grotta, J. C., . . . Hacke, W. (2010). Time to treatment with intravenous alteplase and outcome in stroke: an updated pooled analysis of ECASS, ATLANTIS, NINDS, and EPITHET trials. *The Lancet, 375*(9727), 1695-1703. doi:https://doi.org/10.1016/S0140-6736(10)60491-6

Lees, K. R., Emberson, J., Blackwell, L., Bluhmki, E., Davis, S. M., Donnan, G. A., . . . Zoppo, G. J. d. (2016). Effects of Alteplase for Acute Stroke on the Distribution of Functional Outcomes. *Stroke, 47*(9), 2373-2379. doi:doi:10.1161/STROKEAHA.116.013644

Lehnardt, S. (2010). Innate immunity and neuroinflammation in the CNS: The role of microglia in Toll-like receptor-mediated neuronal injury. *Glia, 58*(3), 253-263. doi:10.1002/glia.20928

Lenz, K. M., & Nelson, L. H. (2018). Microglia and Beyond: Innate Immune Cells As Regulators of Brain Development and Behavioral Function. *Frontiers in Immunology, 9*(698). doi:10.3389/fimmu.2018.00698

Levine, D. A., Galecki, A. T., Langa, K. M., Unverzagt, F. W., Kabeto, M. U., Giordani, B., & Wadley, V. G. (2015). Trajectory of Cognitive Decline After Incident Stroke. *JAMA, 314*(1), 41-51. doi:10.1001/jama.2015.6968

McCarthy, C. A., Vinh, A., Broughton, B. R., Sobey, C. G., Callaway, J. K., & Widdop, R. E. (2012). Angiotensin II type 2 receptor stimulation initiated after stroke causes neuroprotection in conscious rats. *Hypertension, 60*(6), 1531-1537. doi:10.1161/HYPERTENSIONAHA.112.199646

Messé, S. R., Khatri, P., Reeves, M. J., Smith, E. E., Saver, J. L., Bhatt, D. L., . . . Schwamm, L. H. (2016). Why are acute ischemic stroke patients not receiving IV tPA? Results from a national registry. *Neurology, 87*(15), 1565-1574. doi:10.1212/WNL.0000000000003198

Mijajlovic, M. D., Pavlovic, A., Brainin, M., Heiss, W. D., Quinn, T. J., Ihle-Hansen, H. B., . . . Bornstein, N. M. (2017). Post-stroke dementia - a comprehensive review. *BMC Med, 15*(1), 11. doi:10.1186/s12916-017-0779-7

Nogueira, R. G., Jadhav, A. P., Haussen, D. C., Bonafe, A., Budzik, R. F., Bhuva, P., . . . Investigators, D. T. (2018). Thrombectomy 6 to 24 Hours after Stroke with a Mismatch between Deficit and Infarct. *N Engl J Med, 378*(1), 11-21. doi:10.1056/NEJMoa1706442

Perego, C., Fumagalli, S., & De Simoni, M. G. (2011). Temporal pattern of expression and colocalization of microglia/macrophage phenotype markers following brain ischemic injury in mice. *J Neuroinflammation, 8*, 174. doi:10.1186/1742-2094-8-174

Powers, W. J., Rabinstein, A. A., Ackerson, T., Adeoye, O. M., Bambakidis, N. C., Becker, K., . . . Tirschwell, D. L. (2019). Guidelines for the Early Management of Patients With Acute Ischemic Stroke: 2019 Update to the 2018 Guidelines for the Early Management of Acute Ischemic Stroke: A Guideline for Healthcare Professionals From the American Heart Association/American Stroke Association. *Stroke, 50*(12), e344-e418. doi:10.1161/STR.0000000000000211

Rajkovic, O., Potjewyd, G., & Pinteaux, E. (2018). Regenerative Medicine Therapies for Targeting Neuroinflammation After Stroke. *Frontiers in Neurology, 9*(734). doi:10.3389/fneur.2018.00734

Roh, J. S., & Sohn, D. H. (2018). Damage-Associated Molecular Patterns in Inflammatory Diseases. *Immune network, 18*(4), e27-e27. doi:10.4110/in.2018.18.e27

Sag, D., Carling, D., Stout, R. D., & Suttles, J. (2008). Adenosine 5'-monophosphate-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype. *J Immunol, 181*(12), 8633-8641. doi:10.4049/jimmunol.181.12.8633

Shimizu, K., Aikawa, M., Takayama, K., Libby, P., & Mitchell, R. N. (2003). Direct anti-inflammatory mechanisms contribute to attenuation of experimental allograft arteriosclerosis by statins. *Circulation, 108*(17), 2113-2120. doi:10.1161/01.CIR.0000092949.67153.74

Smith, E. E., Saver, J. L., Cox, M., Liang, L., Matsouaka, R., Xian, Y., . . . Schwamm, L. H. (2017). Increase in Endovascular Therapy in Get With The Guidelines-Stroke After the Publication of Pivotal Trials. *Circulation, 136*(24), 2303-2310. doi:doi:10.1161/CIRCULATIONAHA.117.031097

Sorensen, P. S., Lycke, J., Eralinna, J. P., Edland, A., Wu, X., Frederiksen, J. L., . . . investigators, S. s. (2011). Simvastatin as add-on therapy to interferon beta-1a for relapsing-remitting multiple sclerosis (SIMCOMBIN study): a placebo-controlled randomised phase 4 trial. *Lancet Neurol, 10*(8), 691-701. doi:10.1016/S1474-4422(11)70144-2

Srikanth, V. K., Quinn, S. J., Donnan, G. A., Saling, M. M., & Thrift, A. G. (2006). Long-Term Cognitive Transitions, Rates of Cognitive Change, and Predictors of Incident Dementia in a Population-Based First-Ever Stroke Cohort. *Stroke, 37*(10), 2479-2483. doi:doi:10.1161/01.STR.0000239666.46828.d7

Stieler, K., Schumacher, U., Horst, A. K., & Fischer, N. (2012). XMRV induces cell migration, cytokine expression and tumor angiogenesis: are 22Rv1 cells a suitable

prostate cancer model? *PLoS One, 7*(7), e42321. doi:10.1371/journal.pone.0042321

Sun, J. H., Tan, L., & Yu, J. T. (2014). Post-stroke cognitive impairment: epidemiology, mechanisms and management. *Ann Transl Med, 2*(8), 80. doi:10.3978/j.issn.2305-5839.2014.08.05

Thurgur, H., & Pinteaux, E. (2019). Microglia in the Neurovascular Unit: Blood–Brain Barrier–microglia Interactions After Central Nervous System Disorders. *Neuroscience, 405*, 55-67. doi:https://doi.org/10.1016/j.neuroscience.2018.06.046

Walker, K. A., Power, M. C., & Gottesman, R. F. (2017). Defining the Relationship Between Hypertension, Cognitive Decline, and Dementia: a Review. *Curr Hypertens Rep, 19*(3), 24. doi:10.1007/s11906-017-0724-3

Walther, T., Olah, L., Harms, C., Maul, B., Bader, M., Hortnagl, H., . . . Mies, G. (2002). Ischemic injury in experimental stroke depends on angiotensin II. *FASEB J, 16*(2), 169-176. doi:10.1096/fj.01-0601com

Ward, R., Valenzuela, J. P., Li, W., Dong, G., Fagan, S. C., & Ergul, A. (2018). Poststroke cognitive impairment and hippocampal neurovascular remodeling: the impact of diabetes and sex. *Am J Physiol Heart Circ Physiol, 315*(5), H1402-H1413. doi:10.1152/ajpheart.00390.2018

Wattananit, S., Tornero, D., Graubardt, N., Memanishvili, T., Monni, E., Tatarishvili, J., . . . Kokaia, Z. (2016). Monocyte-Derived Macrophages Contribute to Spontaneous Long-Term Functional Recovery after Stroke in Mice. *J Neurosci, 36*(15), 4182-4195. doi:10.1523/JNEUROSCI.4317-15.2016

Wu, F., Luo, T., Mei, Y., Liu, H., Dong, J., Fang, Y., . . . Guo, Y. (2018). Simvastatin alters M1/M2 polarization of murine BV2 microglia via Notch signaling. *J Neuroimmunol, 316*, 56-64. doi:10.1016/j.jneuroim.2017.12.010

Yang, Q., Tong, X., Schieb, L., Vaughan, A., Gillespie, C., Wiltz, J. L., . . . George, M. G. (2017). Vital Signs: Recent Trends in Stroke Death Rates - United States, 2000-2015. *MMWR Morb Mortal Wkly Rep, 66*(35), 933-939. doi:10.15585/mmwr.mm6635e1

Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., . . . Moller, D. E. (2001). Role of AMP-activated protein kinase in mechanism of metformin action. *The Journal of Clinical Investigation, 108*(8), 1167-1174. doi:10.1172/JCI13505