Crucial Role of the Mammalian Target of Rapamycin (mTOR) in Activation of Human Microglia, Mast Cells and Keratinocytes, Inhibitable by Methoxyluteolin

A thesis

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ABSTRACT

Activation of microglia, the resident immune cells of the brain and increased levels of pro-inflammatory cytokines and chemokines have been reported in patients with Autism Spectrum Disorders (ASD); however the stimulus is unknown. Inflammation of the brain in ASD could result from cross-talk between microglia and mast cells (MC), the unique immune cells responsible for allergic and inflammatory processes. ASD risk is associated with gene mutations of regulatory proteins increasing activation of the phosphatidylinositol-3-kinase (PI3K)-dependent mammalian target of rapamycin (mTOR) signaling, and those lowering moesin, an ezrin/radixin/moesin (ERM) cytoskeletal protein. Increased mTOR activity is also evident in MC and in keratinocyte proliferation, resulting in allergies, asthma, mastocytosis or psoriasis, common in mothers having children with ASD.

We had reported the elevated serum levels of the peptide neurotensin (NT) in patients with ASD and psoriasis. We hypothesized that aberrant activation of human microglia, MC and keratinocytes in response to peptides or cytokines will depend on PI3K/mTOR signaling, and will be inhibited by the novel flavonoid methoxyluteolin. We investigated the involvement of PI3K/mTOR activation in neuropeptide or cytokine stimulated cells using specific inhibitors and the natural flavonoids luteolin and methoxyluteolin.

The gene expression and release of the pro-inflammatory mediators were increased for: (a) IL-1β, CXCL8, CCL2 and CCL5 from microglia stimulated by NT or

LPS, (b) TNF, CXCL8 and VEGF from MC stimulated by NT or SP and (c) IL-6, CXCL8 and VEGF from human keratinocytes stimulated by TNF. Methoxyluteolin attenuated mTOR activation, and pro-inflammatory mediator expression and release from stimulated cells. We show that ERM regulate human MC mediator release, and speculate that kinases downstream of mTOR promote moesin phosphorylation, which is also inhibited by methoxyluteolin.

NT-induced human microglia stimulation and which included cell proliferation were mediated via its receptor NTR3/sortilin, which is secreted extracellularly and is uniquely elevated in the serum of ASD patients. Our findings reveal NT as the potential stimulus for activation of human microglia contributing to inflammation of the brain in ASD. Thus, targeting NTR3/sortilin and/or using methoxyluteolin may provide important novel therapeutic options for ASD, and other inflammatory disorders involving abnormal activation of MC and keratinocytes.

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LIST OF ABBREVIATIONS

ASD	Autism Spectrum Disorders (ASD)
AKT	Protein kinase B
BBB	Blood-brain barrier
C1	Complex 1
CCL2	C-C motif chemokine ligand 2/ monocyte chemotactic protein-1
CCL5	C-C motif chemokine ligand 5/normal T-cell expressed and secreted
C-ERMAD	C-terminal ERM association domain
CNS	Central nervous system
CXCL8	C-X-C motif chemokine ligand 8/IL-8
CXCR4	C-X-C motif receptor 4
CRH	Corticotropin-releasing hormone
CRHR	CRH receptor
Cromolyn	Disodium cromoglycate
CSF	Cerebrospinal fluid
C5aR	Complement component 5a receptors
DMSO	Dimethyl sulfoxide
EBP50	ERM-binding phosphoprotein
ELISA	Enzyme-linked immunosorbent assay
ERM	Ezrin/moesin/radixin complex
EZN	Ezrin gene
FBS	Fetal bovine serum
FcεRI	High affinity IgE receptor

FERM	N-terminal Band four-point one, ERM
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCRs	G-protein coupled receptors
GROα	Growth-regulated oncogene
h	Hour/s
HaCaT	Human keratinocyte cell line
HM	Human microglia
HMC-1	Human mast cell-1
H1R/H4R	Histamine receptors
Iba1	Ionizing calcium-binding protein 1
IFN-α	Interferon-α
IgE	Immunoglobulin E
IL	Interleukin
KC	Keratinocyte
KIT	Receptor tyrosine kinase
KU	KU-0063794/dual mTOR complex inhibitor
LPS	Lipopolysaccharides
LTB_4	Leukotriene B_4
LTs	Leukotrienes
LY	LY294002/PI3K inhibitor
Lut	3',4',5,7-tetrahydroxyflavone/luteolin
MC	Mast Cells
Min	Minute/s

mTOR	Mammalian target of rapamycin
Methlut	3’,4’,5,7-tetramethoxyluteolin/methoxyluteolin
MSN	Moesin gene
NF-κB	Nuclear factor-kappa B
NF1	Neurofibromatosis 1
NGF	Nerve growth factor
NHEKs	Normal human epidermal keratinocytes
NK	Neurokinin
NT	Neurotensin
NTR	NT receptor
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PR	Protease-activated receptor
PF	PF 04691502/dual PI3K/mTOR inhibitor
PGD_2	Prostaglandin D_2
PI3K	Phosphoinositide 3-kinases
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
p	Phosphorylation
PP	PP242
PTEN	Phosphatase and tensin homolog
P2X/Y	Purinergic ATP receptors

qRT-PCR	Quantitative real time-polymerase chain reaction
Rap	Rapamycin
RDX	Radixin gene
SCF	Stem cell factor
STAT	Signal transducer and activator of transcription
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment proteins
SP	Substance P
S6K	Ribosomal S6 kinase (S6K)
SORT	Sortilin
SV40	Polyomavirus simian virus 40
TGFβ	Transforming growth factor β
TLR	Toll-like receptors
TNF	Tumor necrosis factor-α
TREM2	Triggering receptor expressed on myeloid cells 2
TSC	Tuberous sclerosis complex
VAMP-8	Vesicle-associated membrane protein 8
VEGF	Vascular endothelial growth factor
4EBP1	4E-binding protein 1

Introduction

An overview of the role of human microglia, mast cells and keratinocytes in the pathogenesis of inflammatory diseases and treatments targeting mTOR signaling

1.1. Cytokine and Chemokine Networks Orchestrate Inflammation

Defining Inflammation: Role of Cytokines & Chemokines

The hallmarks of acute inflammation were initially described over 2000 years ago by the Roman writer Cornelius Celsus by the four cardinal signs: redness, swelling, heat and pain (Tracy, 2006;Rivas, 2010). However, the major understanding of the processes involved in inflammation only began in the 19^{h}-century, and are owed to the milestone discoveries by Rudolph Virchow, Julius Cohnheim and Elie Metchinkoff (Plytycz and Seljelid, 2003). It is has now become clear that the immune system shares mediators and their receptors, with the neuroendocrine system to regulate tissue homeostasis and inflammatory processes, especially those during trauma, infection and injury. These mediators encompass various cytokines and chemokines, which are small signaling peptides (8-11 kDa) produced by both immune and epithelial cells to modulate cellular processes, such as growth, survival, migration and differention, as well as participate in immunosurveillance and immune responses (Rivas, 2010).

Cytokines include, interleukins, interferons, growth factors and chemokines, with pro-inflammatory [tumor necrosis factor-α (TNF), interferon gamma (INF-γ), interleukin (IL) -1β, IL-6, IL-12, IL-17, IL-15, IL-17, chemo-attractants IL-8/CXCL8, monocyte chemotactic protein-1 (MCP-1/CCL2), regulated on activation, normal T-cell expressed and secreted (RANTES/CCL5), angiogenic molecules, such as vascular endothelial growth factor (VEGF)] or anti-inflammatory (IL-4, IL-10, IL-13 and transforming growth factor β (TGFβ) actions. Pro-inflammatory cytokines stimulate both cellular and humoral responses, i.e. activation, adhesion and aggregation of neutrophils, mast cells

(MC), lymphocyte proliferation and antibody production in B cells (Nedoszytko, et al., 2014).TNF and IL-1β are produced during early cellular responses to bacterial pathogens/endotoxins, such as lipopolysaccharide (LPS), which further promote the wave of secondary inflammatory cytokines (IL-6 and IL-17). Chemotactic mediators, such as CXCL8 (IL-8), CCL2 and CCL5 determine the chemical gradient for lymphocyte infiltration towards the site of inflammation (Nedoszytko, et al., 2014).

Disruption of Skin-Blood & Blood-Brain Barriers in Inflammation

The skin is a critical defensive barrier composed of epithelial cells (fibroblasts in the dermis and keratinocytes in the epidermis), endothelial cells and an array of skin-resident immune cells, including T-cells, macrophages, dendritic cells and MC localized near blood and lymphatic vessels. Stimulation by pathogens, chemicals or ultraviolet radiation can lead to release of pro-inflammatory cytokines and chemokines from both epithelial and immune cells, leukocyte infiltration and vascular permeability, resulting in cutaneous inflammation. These processes and cytokines are implicated in the pathogenesis of several skin diseases, such as atopic dermatitis, psoriasis and MC disorders, including systemic mastocytosis (Nickoloff, et al., 2007;Michalak-Stoma, et al., 2011;Nedoszytko, et al., 2014;Theoharides, et al., 2015d).

There is also strong evidence for the existence of a neuroimmunoendocrine circuitry for communication routes between brain and skin immune systems, which has been invoked in several diseases, including those with cutaneous inflammation (Scholzen, et al., 1998;Paus, et al., 2006). Stress is a key stimulus to activate the hypothalamic-pituitary-adrenal (HPA) axis for the secretion of corticotropin-releasing hormone (CRH), adrenocorticotropic hormone and glucocorticoids, as well as an array of

neuropeptides, including nerve growth factor (NGF), neurotensin (NT) and substance P (SP) (Scholzen, et al., 1998;O'Connor, et al., 2004;Theoharides, et al., 2004a;Paus, et al., 2006;Chapman and Moynihan, 2009;Theoharides, et al., 2016a). In fact, both the circulating levels and skin expression of these neuropeptides is elevated in psoriasis and atopic dermatitis (Singh, et al., 1999;Theoharides, et al., 2010a;McGonagle, et al., 2010;Vasiadi, et al., 2012;Vasiadi, et al., 2012a), while the aberrant activation of the skin-resident MC (Harvima, et al., 2008) and keratinocytes (Albanesi, et al., 2007)is also implicated in the disease progression. Importantly both MC (Donelan, et al., 2006;Kulka, et al., 2007;Cochrane, et al., 2011;Asadi, et al., 2012) and keratinocytes (Dallos, et al., 2006;Moura, et al., 2014) express receptors of CRH, NT and SP, stimulation of which could lead to the release pro-inflammatory mediators, including VEGF that increases vascular permeability (Theoharides, et al., 2010a;Li, et al., 2014b;Marina, et al., 2015;Theoharides, et al., 2016a).

Until recently the central nervous system (CNS) was considered "immune privileged", as a consequence of the blood-brain-barrier (BBB) that serves as a shield between the CNS and periphery. The BBB is composed of tight junctions and specialized endothelial cells, the integrity of which can become compromised in the presence of pro-inflammatory cytokines and chemokines (Semple, et al., 2010;Ramesh, et al., 2013;Stuart, et al., 2015). Together with activation of the brain-resident astrocytes, microglia, other glia and MC, as well as the infiltration of peripheral immune cells, the BBB can be compromised, resulting in neuronal damage and focal inflammation of the brain. This has been implicated in several neurodevelopmental disorders, such as Autism

Spectrum Disorders (ASD) (Angelidou, et al., 2012;Hagberg, et al., 2012;Theoharides, et al., 2013a;Bauman, et al., 2014;Le Belle, et al., 2014;Theoharides, et al., 2015c;Theoharides, et al., 2016a)and neurodegenerative diseases(Amor, et al., 2014;Ramesh, et al., 2013;Hong, et al., 2016).

Recent studies have also shown a strong correlation between several autoimmune diseases (including psoriasis) (Gesundheit, et al., 2013) in mothers and the increased risk of ASD developing in their children (Croen, et al., 2005;Kotey, et al., 2014;Zerbo, et al., 2015;Lyall, et al., 2015;Chen, et al., 2015a). Interestingly, the serum levels of the pro-inflammatory peptide NT is elevated in both ASD (Angelidou, et al., 2010;Tsilioni, et al., 2014) and in psoriasis (Vasiadi, et al., 2012a). Numerous pro-inflammatory cytokines and chemokines that could derive from MC and microglia have been reported to be elevated in ASD patients (Zimmerman, et al., 2005;Vargas, et al., 2005;Li, et al., 2009;Tonhajzerova, et al., 2015), while those which could derive from MC and keratinocytes have also been shown to be increased in psoriasis (Michalak-Stoma, et al., 2011;Nedoszytko, et al., 2014;Li, et al., 2016b)**.** Hence, understanding the underlying mechanisms, which can lead to the activation of the brain-resident immune cells, such as MC and microglia or skin-resident cells, including MC and keratinocytes is important to provide therapeutic insights for these inflammatory diseases.

1.2. Microglia and Mast cells (MC)

Microglia: Origins & Expanding Roles in the Brain

Microglia were initially discovered by Pio Del Rio-Hortega in 1919 who revolutionized the study of neuroglia by improving metallic impregnation techniques, which allowed him to study the morphology of these unique brain-resident immune cells. In his book chapter entitled, 'Microglia' (*Cytology and Cellular Pathology of the Nervous System, edited by Wilder Penfield, 1932)*, Del Rio-Hortega first described the morphology of microglia as branched and ramified within the mature brain, while phagocytic invading microglia were more amoeboid (Fig 1.1A). Several studies using genetic tools have revealed that microglia have a distinct lineage and molecular signatures (Salter and Beggs, 2014); microglia arise from the yolk sac as primitive macrophages during embryonic development, where the cells enter the rudimentary brain via the leptomeninges and lateral ventricles by E9.5 and distribute into the CNS even before astrocytes and oligodendrocytes(Kettenmann, et al., 2011;Casano and Peri, 2015;Ginhoux and Prinz, 2015). Under the influence of the brain microenvironment, these amoeboid macrophages differentiate into highly ramified microglia cells (Fig. 1.1) which penetrate into the cerebral cortex via the pial surface, ventricles and choroid plexus (Tay, et al., 2016).

Populations of mature microglia are maintained by self-renewal processes in the healthy CNS (~20 % of cells), where they are crucial for neuronal survival, formation of dendritic spines and pruning of synaptic contacts, i.e. clearance of less-active neurons or apoptotic cells (Paolicelli, et al., 2011;Hughes, 2012;Squarzoni, et al., 2014;Salter and

Beggs, 2014;Casano and Peri, 2015). In adulthood, ramified microglia are highly plastic and contribute to the maintenance and remodeling of the neuronal circuitry (Hughes, 2012;Salter and Beggs, 2014;Chen and Trapp, 2015). Over the course of aging, microglial phenotypes can further diversify (Fig. 1.1B and C) to include hyper-activated, primed and senescent states in the adult human brain, which may potentially contribute to synaptic loss and cognitive decline. Moreover, dysfunction of microglia has also increasingly been associated with the etiology of several neuropsychiatric and neurodegenerative diseases (Prinz and Priller, 2014;von, et al., 2015;Reus, et al., 2015).

Due to their myeloid lineage, microglia have been described to acquire the classical M1 activation, when stimulated by LPS or INFγ, leading to the release of pro-inflammatory mediators, such as IL-1β, IL-6, TNF, CCL2, reactive oxygen species and nitric oxide. These mechanisms for mediator release include the calcium-dependent and lysosomal-regulated exocytosis, as well as secretion via extracellular membrane vesicles involving both exosomes and ectosomes (Prada, et al., 2013). Microglia can also undergo an alternative activation M2 phenotype, which counteracts with the M1 by secretion of the anti-inflammatory cytokine IL-10.

Hence, activation of microglia is also considered important for neuroprotection, since it promotes neurogenesis and suppresses inflammation via release of IL-4, IL-10 and TGF-β (Butovsky, et al., 2014;Chen and Trapp, 2015). In fact, the imbalance of M1 vs. M2 polarized microglial phenotypes within the CNS has recently been implicated in the pathogenesis of neuropsychiatric diseases (Nakagawa and Chiba, 2014) and neuroinflammation (Cherry, et al., 2014).

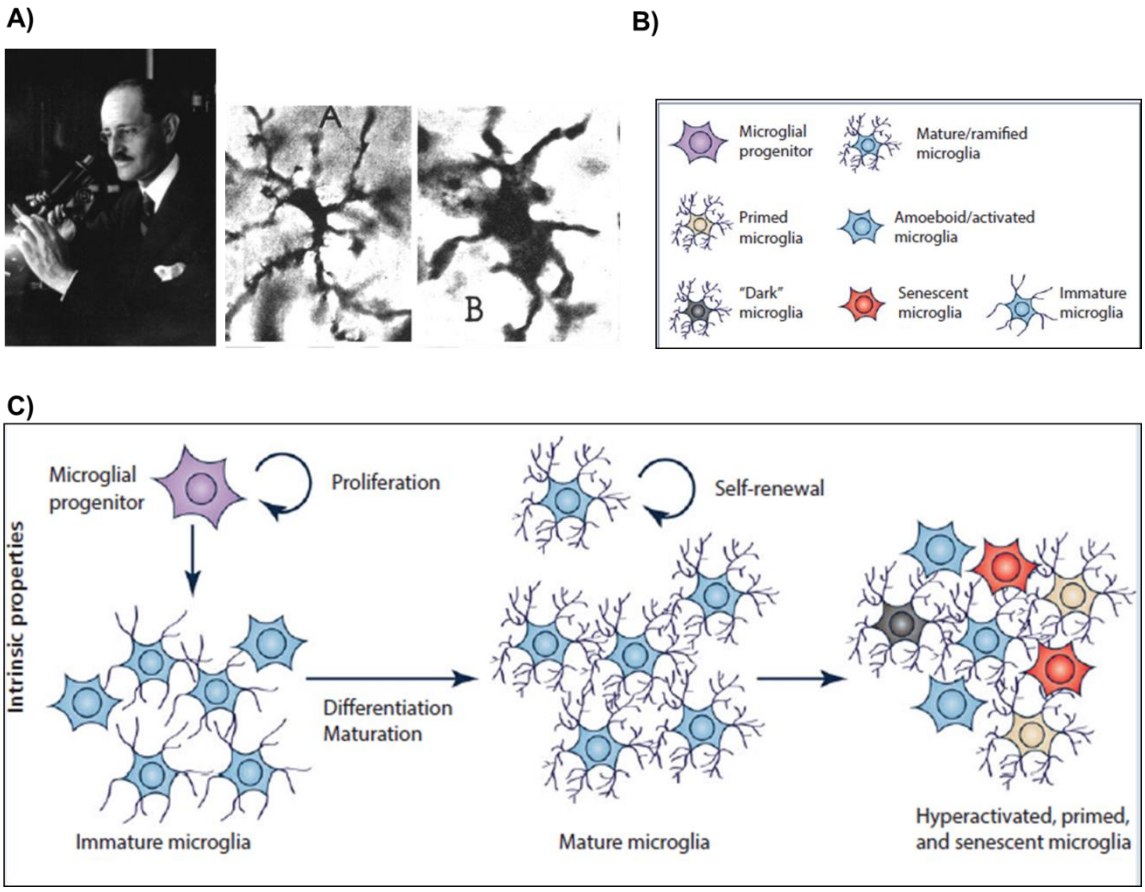


Figure 1.1. Discovery of microglia and their distinct phenotypes. (A) Microglia were discovered by Del Rio-Hortega (1882-1945) by using a silver carbonate staining method. Photomicrographs taken by him are presented here: resting microglia (left) with long and rough prolongations taken from the mature brain; and microglial phagocytic activity (right) with short prolongations and enlarged cell body are shown. (B) It is now known that microglia have multiple phenotypes during development, adulthood and aging. (C) During development, a characteristic increase in the proliferation of immature microglia in the CNS is evident. As microglia mature, their number of processes increase and the cells develop into ramified morphologies, crucial for maintaining neuronal survival and connectivity. Mature microglia can exist as several phenotypes: hyper-activated and primed microglia that are associated with neuroinflammation, while senescent microglia are present in the aging brain [Adapted and modified from (Kettenmann, et al., 2011;Tay, et al., 2016)].

However, it should be noted that classifying microglia in either M1 or M2 polarized states might be an oversimplification, since multiple immune phenotypes of microglia with both M1 and M2 properties have been observed in diseased human brains (Walker and Lue, 2015).

Phenotypic heterogeneity can readily be observed in rodent and human microglia obtained from differing anatomical brain regions (cortex, hippocampus, cerebellum, striatum and spinal cord), which show characteristic differences in surface expression of multiple immune-regulatory proteins, such as cluster of differentiation protein 11b (CD11b) or integrin αM, CD40, CD45, CD80, CD86, ionizing calcium binding protein 1 (Iba1), F4/80 and triggering receptor expressed on myeloid cells 2 (TREM2) (Olah, et al., 2011). The expression of these marker proteins, such as Iba1is associated with activation of rodent microglia (Nikodemova and Watters, 2012), but it does not reflect the pro-inflammatory phenotype of human microglia (Walker and Lue, 2015). Recent studies have further revealed that the expression of receptors for inflammatory stimuli, such as IFNγ and LPS in human microglia is very low or absent, compared to rodent microglia (Smith and Dragunow, 2014), suggesting inter-species related differences between microglia.

Human microglia express membrane receptors for several neurotransmitters and neuropeptides, thus allowing communication with neurons (Pocock and Kettenmann, 2007;Kettenmann, et al., 2011;Kettenmann, et al., 2013;Pannell, et al., 2014), astrocytes (Pascual, et al., 2012) and MC (Skaper, et al., 2012a;Frick, et al., 2013;Skaper, et al., 2014a;Skaper, et al., 2014b). Cross-talk between MC and microglia is now also

considered important in inflammation of the brain (Skaper, et al., 2012a;Skaper, et al., 2014b;Dong, et al., 2014b).

Mast cells: Regulators of Inflammation and Immunity

Mast cells (MC), together with basophils were first characterized by the Nobel-prize winning immunologist Paul Ehrlich in 1878/79 (Blank, et al., 2013). MC have distinct histochemical and morphological properties, including the striking presence of cytoplasmic granules (Fig. 1.2A), with cell sizes ranging from 6-12 μM (Beaven, 2009). Human MC originate from hematopoietic precursors ($CD34^+/CD117^+$) in the bone marrow, which circulate in the blood and mature in vascularized tissues (Rodewald, et al., 1996;Chen, et al., 2005a;Williams and Galli, 2000;Galli, et al., 2008b;Theoharides, et al., 2012a). Mature MC strategically reside at sites encountering environmental stimuli (antigens, allergens, peptides, and endotoxins), such as upper dermal skin, respiratory mucosa, gastrointestinal tract and on the brain side of the BBB (Theoharides, et al., 2012b;Silver and Curley, 2013).

Although traditionally considered important for their role in immunoglobulin type E (IgE)-associated allergic processes via cross-linking of their high affinity surface receptors (FcεRI)(Galli, 1993;Wedemeyer, et al., 2000;Rivera, et al., 2008;Galli and Tsai, 2012;Galli, 2016;Modena, et al., 2016) (Fig. 1.2B), numerous studies have revealed that MC are involved in both innate and acquire immunity (Galli, et al., 2005;Galli, et al., 2008a;Cardamone, et al., 2016), as well as in inflammation (Lawrence, et al., 2002;Harvima and Nilsson, 2011;Theoharides, et al., 2012b;Dong, et al., 2014b;Theoharides, et al., 2015d).

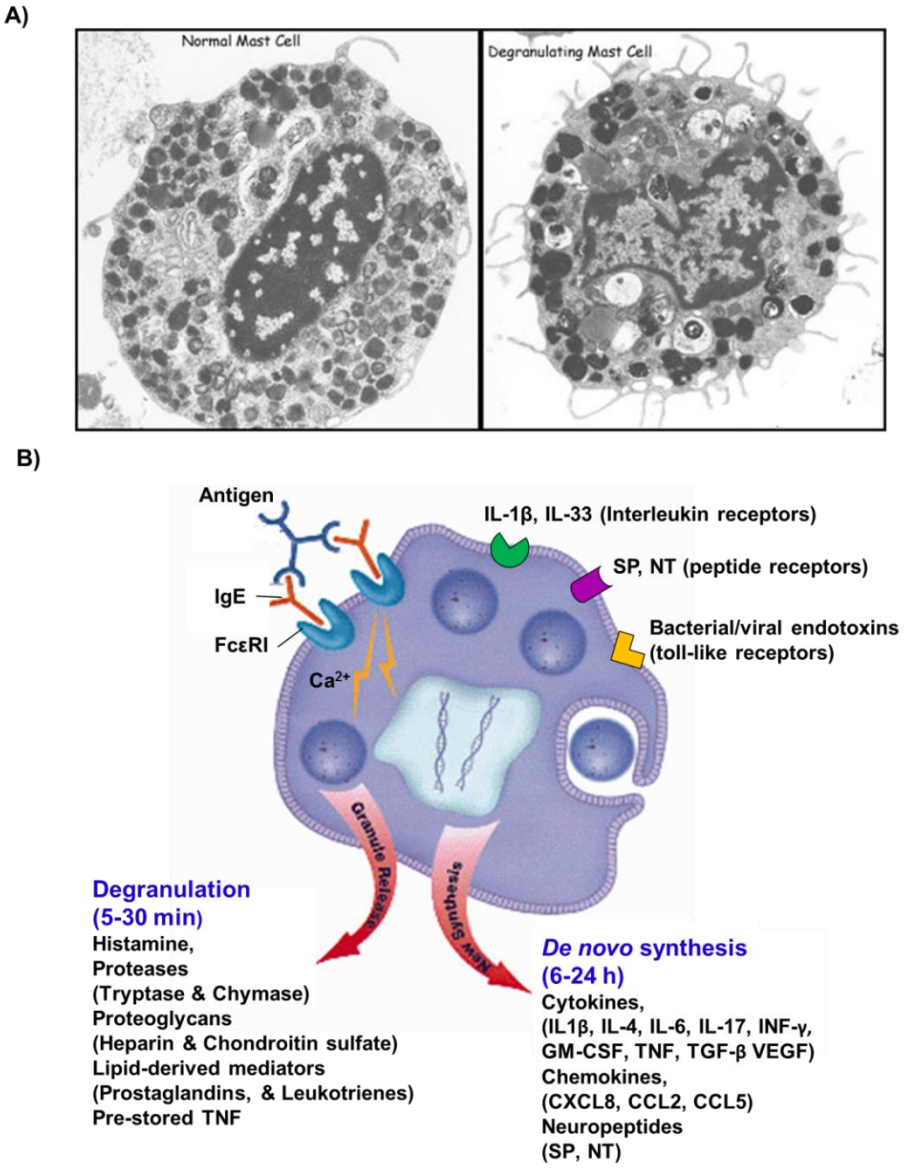


Figure 1.2. Electron microscopic images of isolated tissue MC and schematic of MC stimuli and mechanisms of mediator release. (A) Note the presence of the prominent and numerous cytoplasmic granules in the resting MC (left). Upon degranulation, MC release their granular contents (right), which contain varying compositions of pre-stored mediators. (B) Stimulation of MC by various stimuli, as shown produce a vast array of mediators, which include biogenic amines, cytokines, enzymes, lipid metabolites, neuropeptides, growth factors and ATP. [Figure adapted from Theoharides laboratory (unpublished)].

Activation of MC is denoted as biphasic responses: (a) MC degranulation (0.5- 2 h) characterized by the immediate release of granular contents, including histamine, proteoglycans (heparin, chondroitin sulfate and hyaluronic acid), proteases (tryptase and chymase), lipid-derived mediators (leukotriene B_4 (LTB_4), LTC_4, prostaglandin D_2 and platelet activating factor)(Boyce, 2007;Metcalfe, 2008) and pre-stored cytokine, tumor necrosis factor (TNF) (Zhang, et al., 2012a); (b) MC release of *de novo* synthesized mediators (6-24 h), including cytokines [interferon-α (IFN-α), interleukin (IL)-1β, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13,IL-17, granulocyte-macrophage colony-stimulating factor (GM-CSF), transforming growth factor β (TGF β), TNF and vascular endothelial growth factor (VEGF)] and chemokines [(C-C motif) ligand 2 (CCL2), CCL5, CXCL8](Theoharides and Cochrane, 2004a;Theoharides, et al., 2015d), which can be occur selectively without degranulation(Theoharides, et al., 2007b)(Fig. 1.2B).

The ability to release multiple mediators allows MC to actively interact with other cell types in their surrounding environment, and participate in the induction and/or propagation of various immune and inflammatory responses. Abnormal activation of MC is now implicated in several diseases, including mastocytosis (Metcalfe and Akin, 2001;Petra AI, et al., 2014;Theoharides, et al., 2015d), asthma (Galli and Tsai, 2012;Sismanopoulos, et al., 2013), atopic dermatitis, (Vasiadi, et al., 2012), psoriasis (Sismanopoulos, et al., 2013;Maurer, et al., 2003;Castells, 2006;Theoharides, et al., 2012b) and autism spectrum disorders (ASD) (Theoharides T.C., et al., 2013a;Theoharides T.C., et al., 2016b).

Presently, the only clinically available MC stabilizer, disodium cromoglycate (cromolyn) is ineffective for inhibition of human MC (Oka, et al., 2012;Vieira Dos, et al., 2010), while cromolyn treatment in patients can lead to severe side-effects, such as tachyphylaxis (Theoharides, et al., 1980) or contact dermatitis (Camarasa, et al., 1997;Kudo, et al., 1988). Another MC stabilizer and histamine receptor 1 (H1R) antagonist that has been used to relieve symptoms of irritable bowel syndrome (Klooker, et al., 2010) and fibromyalgia (Ang, et al., 2014), which involve MC activation, only weakly inhibits histamine and prostaglandin release and is associated with side-effects, such as weight gain, irritability and increased nose-bleeds. Other inhibitors of MC mediators, which include anti-TNF biologics and histamine or protease or leukotriene antagonists are associated with increased risk of infections, and more recently blood malignancies (Finn and Walsh, 2013). Hence, understanding the regulation of MC is crucial for identifying novel molecular targets and therapies (Harvima, et al., 2014) for inhibition of MC.

Communication between Microglia and MC: Relevance to Inflammation of the Brain

There is substantial evidence that indicates that in vertebrates, including humans, MC are present in the brain, on the brain side of the BBB and in the leptomeninges, thalamus, hypothalamus and in the dura mater (Dropp, 1976;Dropp, 1979;Theoharides, 1990;Silver, et al., 1996;Florenzano and Bentivoglio, 2000;Kandere, et al., 2001). MC lie in close proximity to the basal side of brain blood vessels and are able to communicate with other brain-resident cells, such as neurons, astrocytes and microglia (Silver and Curley, 2013;Nakao, et al., 2015). Despite their considerably smaller numbers than neurons and microglia, activated MC have the ability to modulate the BBB permeability

(Ribatti, 2015), while mature MC can also migrate from the blood to brain (Silver, et al., 1996;Silverman, et al., 2000). Several MC mediators, such as histamine, serotonin, matrix metalloproteinases, nitric oxide, calcitonin gene-related peptide**,** vasoactive intestinal peptide and cytokines, including TNF and VEGF can increase vascular permeability, leading to BBB disruption (Theoharides, et al., 2012b;Dong, et al., 2014b). In fact, these MC effects on vascular permeability are blocked by the MC stabilizer cromolyn and are absent in MC deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice **(**Theoharides, et al., 2005).

Cross-talk between MC and microglia is now considered important in inflammation of the brain (Skaper, et al., 2012;Skaper, et al., 2013;Skaper, et al., 2014a;Dong, et al., 2014b). MC-derived histamine not only regulates the BBB permeability (Gross, 1982), but can also stimulate the activation of microglia via its histamine receptors H1R and H4R to release pro-inflammatory IL-6 and TNF (Dong, et al., 2016). Similarly, MC-derived tryptase can cleave and activate protease-activated receptor 2 (PAR2) on microglia (also resulting in upregulation of purinergic receptor P_2X_4 and release of brain-derived neurotrophic factor (BDNF) from microglia (Yuan, et al., 2010), while microglia-derived IL-6 and TNF upregulates PAR2 expression on MC and TNF release (Zhang, et al., 2016b) implicated in neuroinflammation(Gu, et al., 2015). Bidirectional cross-talk between MC and microglia can occur via numerous mediators and mechanisms involving the (a) toll-like receptors (TLR2 and TLR4), (b) purinergic ATP receptors (P_2X and P_2Y), (c) histamine receptors (H1R and H4R), (d) protease-activated receptor 2 (PAR2), (e) complement component 5a receptors (C5aR) and (f) chemokine (C-X-C motif) receptor 4 (CXCR4) (See Table 1) (Skaper, et al., 2012;Skaper, et al., 2013;Silver and Curley, 2013).

TABLE 1.1. Potential avenues for mast cell-microglia communication

Effector	Biological Actions	
	Mast Cells (MC)	Microglia
Toll-like receptors (TLR2/TLR4)	Upregulation of cytokine/chemokine release; CCL5/RANTES induces pro-inflammatory microglia; recruitment of immune cells to site	Release of IL-6 and CCL5 affects surface expression of TLR2/TLR4 on MC
ATP receptors (P_2X and P_2Y)	IL-33 binds to MC and induces expression of IL-6, IL-13 and CCL2, which in turn modulates microglial activation	ATP stimulates IL-33 release from microglia pre-activated with PAMPS via TLR
Histamine receptors (H1R/H4R)	Microglia-derived IL-6 and TNF induces expression of histamine and cytokines in MC	MC histamine activate microglia via H1R and H1R to release TNF and IL-6
Proteinase-activated receptor 2 (PAR2)	Microglial-derived IL-6 and TNF up-regulate MC expression of PAR2, resulting in MC activation and TNF release	MC tryptase cleaves and actives PAR2 on microglia resulting in P2X4 upregulation and BDNF release
C5a receptor (C5aR) C5a and TLR4 cross-talk	C5aR up-regulated upon MC activation; C5a peptide is a strong MC chemoattractant	C5aR up-regulated upon microglia activation; Release C5a peptide
CXCR4 receptor /CXC12 chemokine	CXCR4 acts as a MC chemotaxin	CXCR4 promotes microglia activation and migration; CXCR4/CXC12 is upregulated

PAMPS; pathogen associated molecular patterns
(Adapted and modified from Skaper, et al. 2012; 2013)

In vivo studies have further revealed that MC-induced activation of microglia is blocked by cromolyn or is absent in MC deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice (Dong, et al., 2016),while in another study cromolyn treatment in rats inhibited activation of brain MC that induced microglial release of IL-6 and TNF and decreased neuronal damage, as well as improved cognition function in these animals(Zhang, et al., 2016a). These recent studies further provide evidence that brain MC can now be evoked in the activation of microglia, all of which is implicated in neuroinflammation. The endogenous neuroprotective lipid mediator N-palmitoylethanolamine,which is synthesized and

hydrolized by microglia has been reported to down-regulate activation of MC (Skaper, et al., 2013). Hence, the pharmalogical attenuation of microglial and MC activation is emerging as an attractive therapeutic avenue for inflammation of the brain.

1.3. Autism Spectrum Disorders (ASD)

Implications of MC and Microglia Activation in ASD

Considerable evidence indicates that inflammation of the brain during fetal and perinatal life (Angelidou, et al., 2012;Hagberg, et al., 2012;Hagberg, et al., 2015) contributes to the pathogenesis of neurodevelopmental disorders (Chavarria and Alcocer-Varela, 2004;Jones and Thomsen, 2013;Hong, et al., 2016), such as ASD (Theoharides, et al., 2013a;Le Belle, et al., 2014). For instance, circulating auto-antibodies for fetal brain proteins have been detected in maternal blood (Zimmerman, et al., 2007;Braunschweig and Van de Water, 2012) and in some ASD patients (Rossi, et al., 2011), suggesting that a disruption of the fetal BBB may occur during pregnancy. Moreover, the presence of auto-fetal brain antibodies in ASD patients was shown to significantly correlate with allergic symptoms (Mostafa and Al-Ayadhi, 2013), implying the activation of MC. MC-derived mediators, such as VEGF could disrupt the BBB, thus contributing to inflammation of the brain and the pathogenesis of ASD (Theoharides, et al., 2013a).

In fact, modeling maternal infection in mice via administration of LPS or the viral double-stranded RNA poly (I:C), described as MIA results in offspring with immune dysregulation and ASD-like behavior (Hsiao and Patterson, 2011;Hsiao, et al.,

2012;Garay, et al., 2013). In MIA, the increased levels of maternal IL-6 (Smith, et al., 2007) or IL-17A (Garay, et al., 2013) have been identified as the key mediators in yielding the core behavioral and neuropathological ASD-like symptoms. Importantly, targeting inhibition of T helper 17 (T_h17) cells, which predominantly secrete IL-17A or using antibody blocked of IL-17A activity in pregnant MIA mice is protective against behavioral abnormalities (Choi, et al., 2016). Interestingly, MC also secrete IL-17A (Chen, et al., 2015b), while MC-derived IL-6 and TGFβ promotes the development of T_h17 cells (Ye, et al., 2014). In addition, MC-derived TNF can promote T_h17-dependent neutrophil recruitment (Nakae, et al., 2007).

The abnormal activation of microglia has also been reported in brains of patients with ASD (Vargas, et al., 2005;Rodriguez and Kern, 2011;Morgan, et al., 2010;Morgan, et al., 2014;Edmonson, et al., 2014). Microglia are important during the healthy brain development because they "prune" neural circuits (Paolicelli, et al., 2011;Schafer, et al., 2012;Casano and Peri, 2015). Microglia not only regulate neurogenesis and synaptic maturation at perinatal and postnatal stages (Squarzoni, et al., 2014), but also clear synaptic traffic during brain development (Bilimoria and Stevens, 2015) to maintain proper neuronal function (Chen and Trapp, 2015). Dysfunction of microglia may result in choking" of normal synaptic traffic and lead to inflammation of the brain. This is evident from a recent transcriptome analysis of human brain tissue from patients with ASD, which identified gene clusters associated with increased microglia activation and decreased neuronal function (Gupta, et al., 2014).

Numerous pro-inflammatory cytokines and chemokines, which could derive from MC and microglia, such as IL-1β, IL-6, TNF, CXCL8 and CCL2 have been reported to

be elevated in the brain and cerebrospinal fluid of ASD patients (Zimmerman, et al., 2005;Vargas, et al., 2005;Li, et al., 2009). Moreover, the increased plasma/serum levels of IL-1β, IL-6, CXCL8, CCL2 and CCL5 in children with ASD were reported to be positively correlated with impaired communication and aberrant behavior (Ashwood, et al., 2011;Masi, et al., 2015). As a result, microglia are now considered an important component in the pathogenesis of ASD (Takano, 2015;Koyama and Ikegaya, 2015;Kern, et al., 2015;Young, et al., 2016).

Elevated neurotensin in ASD, a potential stimulus for activation of microglia and MC

Previously, our laboratory reported the elevated levels of serum neurotensin (NT), a gut and brain peptide (Carraway and Leeman, 1973;Dobner, et al., 1987;Dobner, 2005) in children with ASD (Angelidou, et al., 2010;Tsilioni, et al., 2014). In the CNS and brain, NT is primarily secreted from neurons (Dobner, et al., 1987) and astrocytes (Vincent, et al., 1994). NT can modulate the dopaminergic and glutamate/GABAergic neurotransmitter systems in the CNS (St-Gelais, et al., 2006) and can increase vascular permeability (Donelan, et al., 2006), which has been implicated in pathogenesis of ASD (Ghanizadeh, 2010;Boules, et al., 2014).

Intriguingly, NT receptors in the human brain transiently increase after birth (Zsurger, et al., 1992) and are more concentrated in the hypothalamus (Najimi, et al., 2014) and the amygdala (Lantos, et al., 1996), which regulate behavior associated with the symptomatology of ASD. NT responses are mediated through three receptors: NTR1 and NTR2, which belong to the G protein-coupled receptor family (Vincent, 1995;Vincent, et al., 1999) and NTR3/sortilin (~110 kDa) , a type I sorting receptor belonging to the vacuolar protein sorting 10 protein (Vps10p) domain family(Petersen, et

al., 1997;Westergaard, et al., 2004). Sortilin is a multifaceted receptor/co-receptor implicated in neuronal survival and apoptosis (Nykjaer and Willnow, 2012). Interestingly, NTR3/sortilin is mainly expressed in the CNS during embryonic development (Hermans-Borgmeyer, et al., 1999), and has been found to be the only NT receptor expressed in murine microglia (Dicou, et al., 2004) and an immortalized human microglia cell line (Martin, et al., 2005).

The extracellular domain of sortilin (termed soluble sortilin ~26 residues) is released by proteolysis from the cell surface of neurons (Chen, et al., 2005b) and other cell types (Carlo, et al., 2014). A recent study reported the increased serum levels of sortilin in patients with depression, which were associated with increased serum levels of BDNF and VEGF (Buttenschon, et al., 2015). Sortilin serves as (a) a scavenger receptor in hepatocytes and adipocytes and is implicated in cholesterol metabolism and obesity (Dube, et al., 2011), (b) a sorting receptor, regulating intracellular BDNF and its secretory pathway in neurons (Chen, et al., 2005b;Wilson, et al., 2014) and (c) a single-transmembrane receptor or co-receptor for ligands, such as NT (Navarro, et al., 2001) or apolipoprotein E (Carlo, 2013).

Several studies have also shown that MC can be activated by NT (Lazarus, et al., 1977a;Lazarus, et al., 1977b;Kulka, et al., 2007). NT stimulates rodent peritoneal MC to secrete histamine (Carraway, et al., 1982;Feldberg, et al., 1998;Barrocas, et al., 1999) and has been shown to induce the expression of CRH receptor-1 in human MC, through which NT synergistically with CRH stimulates VEGF release MC (Donelan, et al., 2006). MC express the NT receptor 1 and synthesize precursor NT peptides (Cochrane, et al.,

2011), as well as MC degrade NT (Piliponsky, et al., 2008), indicating their role in the regulation of circulating NT levels.

ASD and its Associated Risk Factors

ASD are a heterogeneous group of pervasive neurodevelopmental disorders characterized by stereotypic or repetitive behaviors and deficits in language, intelligence and social communication (Fombonne, 2009;Lai, et al., 2014;Volkmar and McPartland, 2014). In the US, ASD is now estimated to be 1 in 45 children (Developmental Disabilities Monitoring Network Surveillance Year 2010 Principal Investigators Centers for Disease Control and Prevention (CDC), 2014;Zablotsky B and et al., 2015), while the global prevalence of ASD remains to be determined (Elsabbagh, et al., 2012). The lack of reliable biomarkers for ASD diagnosis (Ruggeri, et al., 2014) or knowledge in distinct mechanisms for disease pathogenesis (Willsey and State MW, 2015) has hampered the development of ASD treatments (Ghosh, et al., 2013). Hence, ASD is a major public health problem, resulting in a total annual economic burden cost of $268 billion in 2015 and projected healthcare costs to reach $416 billion in 2025 (Leigh and Du, 2015).

Initially described by Kanner in the 1943 as “early infantile autism” (Kanner, 1943), researchers believed that this disorder was a consequence of emotional deprivation and a psychiatric condition. This notion has now been discarded, and rather ASD is now recognized to involve immune and neuronal dysfunction (Zimmerman, et al., 2005;Li, et al., 2009;Rossignol and Frye, 2012;Bauman, et al., 2014;Estes and McAllister, 2015;Young, et al., 2016), as well as inflammation of the brain (Hagberg, et al., 2012;Theoharides, et al., 2013a;Theoharides, et al., 2015c). In addition, various environmental factors have also been implicated in the risk of developing ASD (Ming, et

al., 2008;Herbert, 2010;Goines and Ashwood, 2012;Rossignol, et al., 2014;Wong, et al., 2015).

In fact, administration of LPS or the viral double-stranded RNA poly (I:C), results in offspring with immune dysregulation and ASD-like behavior (Hsiao and Patterson, 2011;Hsiao, et al., 2012;Garay, et al., 2013). Recent studies have also shown the strong associations between several maternal autoimmune diseases (including asthma, allergies and psoriasis) (Gesundheit, et al., 2013) and ASD (Croen, et al., 2005;Zerbo, et al., 2015;Lyall, et al., 2015;Kotey, et al., 2014;Chen, et al., 2015a). Hence, accumulating evidence indicates that immune dysfunction in the brain and periphery could contribute to the pathophysiology of ASD (Estes and McAllister, 2015).

ASD is also known to involve a complex genetic basis, including rare copy number variants and single-gene mutations that account for ~15% of ASD cases (Kelleher, III and Bear, 2008;Shen, et al., 2010;Schaaf and Zoghbi, 2011). A subset (1-5%) of these single-gene mutations in tuberous sclerosis complex (TSC) 1/2, neurofibromatosis 1 (NF1) and phosphatase and tensin homolog (PTEN) can lead to syndromic forms of ASD with tuberous sclerosis, neurofibromatosis, or macrocephaly, respectively (Kelleher, III and Bear, 2008;Bourgeron, 2009;Hoeffer and Klann, 2010;Sawicka and Zukin, 2012).

The encoded TSC1/TSC2, NF1 and PTEN proteins negatively regulate the mammalian target of rapamycin (mTOR) signaling, which is activated by the phosphoinositide-3 kinase (PI3K) pathway (Figure 1.3). Interestingly, *Pten*-knockout mice exhibit ASD-like behavior with cognitive impairments and deficits in social interactions (Kwon, et al., 2006). More recently, a dysregulated downstream mTORC1 translational control, evident in *Eif4ebp2*-knockout mice has also been linked to an ASD-like phenotype (Gkogkas, et al., 2012;Wang and Doering, 2013), implicating hyper-active mTOR signaling in ASD pathogenesis.

1.4. Mammalian target of rapamycin (mTOR) signaling

Structure and Function of mTOR Kinase

The phosphatidylinositol-3-kinase (PI3K)-dependent mammalian target of rapamycin (mTOR) is a crucial signaling hub, integrating environmental stimuli with cellular processes for normal homeostasis (Laplante and Sabatini, 2012), while abnormalities in this pathway result in severe defects in brain developmental processes (Lee, 2015;Lipton and Sahin, 2014). mTOR serves as the catalytic subunit in two large complexes: (a) mTORC1, consists of six known protein components (mTOR, Raptor, mLST8/GβL, DEPTOR, PRAS40, tel2 and tti) and is involved in mRNA translational control by phosphorylation of downstream substrates, the ribosomal S6 kinases (S6K1/2) and the eukaryotic initiation factor 4E-binding proteins (4EBP 1/2/3), and (b) mTORC2 , consists of seven known protein components (mTOR, Rictor, Protor, DEPTOR,

mLST8/GβL, mSIN1, tel2 and tti) implicated in cytoskeleton reorganization (Laplante and Sabatini, 2012) (Fig. 1.3).

Many insights into mTOR signaling have come from investigations using the action of rapamycin (sirolimus), a bacterial macrolide and allosteric inhibitor that binds FKB12 receptor (Fig. 1.3A), after which the kinase was named (Paghdal and Schwartz, 2007;Laplante and Sabatini, 2012). Rapamycin has had diverse clinical applications as an anti-fungal, immunosuppressant and anti-cancer drug (Sehgal, 2003;Paghdal and Schwartz, 2007). However, rapamycin only potently inhibits phosphorylation of the S6Ks, but is resistant in blocking 4EBP1 phosphorylation and hence, cap-dependent protein translation (Choo, et al., 2008;Thoreen, et al., 2009). To counteract this, rapamycin is often combined with the ATP-competitive PI3K inhibitor, LY294002 (Takeuchi, et al., 2005) or clinically available dual PI3K/mTOR inhibitor PF-04691502 (Yuan, et al., 2011). Recently, a new generation of potent dual mTORC1/mTORC2 inhibitors, including KU 0063794 (Garcia-Martinez, et al., 2009), PP242 and Torin 1(Zhou, et al., 2010) have been employed to decipher the functional roles of mTORC1 versus mTORC2 signaling.

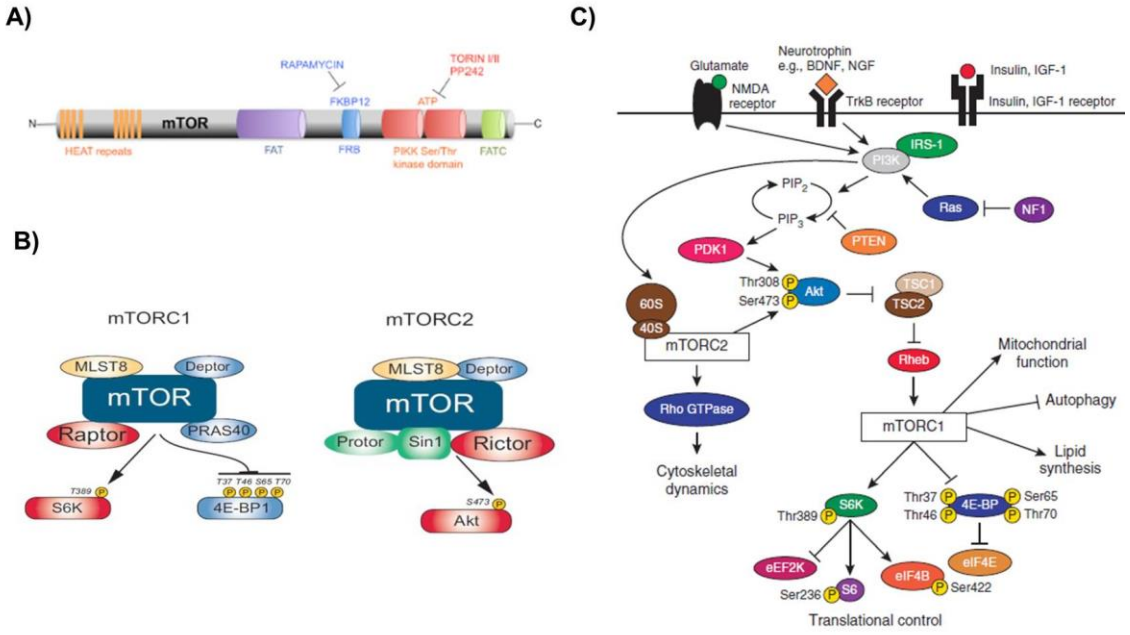


Figure 1.3. Domain structure of the mTOR kinase, its protein components and regulation of the signaling pathway. (A) Domain organization of the mTOR kinase, showing HEAT repeats, which mediate protein interactions with Raptor and Rictor; FKB12-rapamycin binding domain (FRB), the site for binding to rapamycin to inhibit mTORC1; PIKK kinase domain that contains the Ser/Thr catalytic activity, the site for inhibition mediated by Torin 1 and PP242. (B) The components of mTORC1 and mTORC2 include: mTOR, the catalytic subunit; Raptor, scaffolding protein essential for mTORC1 activity and rapamycin sensitivity; PRAS40 and DEPTOR, the inhibitory mTORC1 proteins; mLSTB/GβL; Rictor, scaffold protein essential for mTORC2; mSIN1, crucial for AKT phosphorylation; Protor. (C) mTORC1 is activated by receptor signaling via the PI3K/AKT pathway. mTORC1 regulates protein synthesis via its downstream substrates, the 4EBP 1/2/3 and S6K1/2. mTORC2 regulates actin cytoskeleton dynamics via Rho GTPase and PKCα/θ [Adapted and modified from (Costa-Mattioli and Monteggia, 2013;Lipton and Sahin, 2014)].

Hyper-active mTOR Signaling and Aberrant Activation of Microglia, MC and Skin-resident Keratinocytes

The differentiation, proliferation and activation of immune cells, such as neutrophils, dendritic cells, macrophages (Saemann, et al., 2009), T- and B- lymphocytes (Goh, et al., 2013;Delgoffe and Powell, 2009;Xie, et al., 2014) and MC (Kim, et al., 2008a;Smrz, et al., 2011;Kuehn, et al., 2011;Halova, et al., 2012;Weichhart and Saemann, 2008) is regulated by the PI3K/mTOR signaling pathway. The mTORC1 signaling has been reported to regulate the cytokine expression in cultured murine microglia in response to the bacterial endotoxin LPS (Dello, et al., 2009), while inhibitors of mTOR prevent the induction of the anti-inflammatory M2 activation phenotype (Zhong, et al., 2012;Lisi, et al., 2014). In a rodent model of neuroinflammation, mTOR signaling has been shown to promote the pro-inflammatory M1 phenotype and IL-1β release (Li, et al., 2016a).

The PI3K/mTOR signaling pathway is also important for the normal homeostasis of human MC (Kim, et al., 2008a) and those following FcεRI-mediated allergic responses (Smrz, et al., 2011). Moreover, recent studies have further shown that while mTORC1 primarily regulates normal human MC homeostasis, mTORC2 mediates the survival and proliferation of neoplastic MC in diseased states (Smrz, et al., 2011) and is crucial for MC chemotaxis (Kuehn, et al., 2011;Halova, et al., 2012). MC derived from TSC1-deficient mice, with increased mTOR activation have impaired degranulation and release increased levels of pro-inflammatory IL-6 and TNF in response to FcεRI-mediated allergic stimulation (Shin, et al., 2012). Moreover, deficiency in PTEN, also increases

activation of MC (Furumoto, et al., 2006) and can lead to a mastocytosis-like state, resulting from increased proliferation of MC (Furumoto, et al., 2011).

In addition, the PI3K/AKT/mTOR pathway also plays a pivotal role in the proliferation of epidermal keratinocytes (Mitra, et al., 2012) and keratinocyte activation in response to NGF (Zhang and Ma, 2014) or the 1L-17A and IL-22 cytokines (Datta, et al., 2013;Raychaudhuri and Raychaudhuri, 2014) to regulate expression of VEGF and angiogenesis. In fact, pro-inflammatory cytokines produced by the polarized subsets of T-cells and MC, which include TNF, INFγ, IL-17A, IL-22, IL-23 have been associated with the onset of psoriasis by inducing the aberrant activation of keratinocytes (Nestle, et al., 2009;Grozdev, et al., 2014). The expression of phosphorylated mTOR and its downstream substrates S6K is also elevated in lesional psoriatic skin (Choi, et al., 1997;Buerger, et al., 2013;Raychaudhuri and Raychaudhuri, 2014), further suggesting a role of mTOR signaling in the pathogenesis of psoriasis. In fact, the dual PI3K/mTOR kinase inhibitor NVP-BEZ235 has potent anti-mitotic effects on keratinocyte (Raychaudhuri and Raychaudhuri, 2014) and on MC proliferation and activation (Blatt, et al., 2012) may provide a treatment option for psoriasis. Recently it was also shown that the clinically available mTOR inhibitors, sirolimus and everolimus block proliferation of keratinocytes and alter pro-inflammatory cytokine production (DeTemple, et al., 2016).

1.5. Ezrin-Radixin-Moesin (ERM) Cytoskeletal Proteins

Discovery of ERM and tissue-specific functions

The ezrin, radixin and moesin (ERM) belong to a family of highly homologous proteins that provide a regulated linkage between filamentous (F)-actin in the cell cortex and membrane proteins on the surface of cells (Algrain, et al., 1993;Bretscher, 1999;Tsukita and Yonemura, 1999;Tsujita and Itoh, 2015). This regulated attachment of F-actin to the plasma membrane is essential for several fundamental cellular processes, including determination of cell shape, polarity and surface structures, cell adhesion, cell motility, phagocytosis, as well as for the integration of signal transduction with mediator trafficking and membrane transport (Shaffer, et al., 2009;Bretscher, et al., 2002;Charrin and Alcover, 2006;Fehon, et al., 2010;Neisch and Fehon, 2011;Pore and Gupta, 2015;Tachibana, et al., 2015). Many insights into ERM functions have come from studies in lower organisms, such as the *Drosophila*, which harbors a single ERM gene for the encoded protein moesin that is essential for viability and epithelial integrity (Bretscher, et al., 2002). In mammals, ERM are encoded by three genes that each give rise to a single protein, which maintain a high degree of structural conservation (Fig. 1.4A).

Ezrin was initially identified as a component of cell surface structures within microvilli and membrane ruffles in epithelial cells (Bretscher, 1983), while radixin was later characterized as an F-actin capping protein, isolated from adherens junctions (Tsukita and Hieda, 1989). Moesin, very closely related to ezrin, was identified as a protein that binds to heparin, a glycosaminoglycan (Lankes, et al., 1993;Lankes and Furthmayr, 1991). ERM are widely expressed, but their relative expression varies among

different tissues, with at least two proteins present in numerous cell types. For instance, ezrin is found primarily in epithelial cells (Berryman, et al., 1993), while moesin is the predominant ERM in endothelial cells, platelets (Nakamura, et al., 1995;Nakamura, et al., 1996;Nakamura, et al., 1999) and MC (Doi, et al., 1999). Ezrin and moesin are the only proteins found to be co-expressed in human monocytes/macrophages, neutrophils and lymphocytes (Fehon, et al., 2010). Hence, ERM can often be functionally redundant in the regulation of cellular processes.

Structural Organization and Activation of ERM

ERM are structurally related by virtue of their shared N-terminal Band four-point one, ERM (FERM), where ~300 residues are implicated in binding to the plasma membrane proteins (Chishti, et al., 1998;Yonemura, et al., 1998) and ~80 residues of the C-terminal end are involved in association with F-actin (Frame, et al., 2010) (Fig. 1.4A). Several X-ray structures of the isolated FERM of ERM have been solved independently (Fehon, et al., 2010;Frame, et al., 2010), which show that the FERM domain is composed of three sub-domains arranged like a clover leaf. Conversely, the structure full-length insect moesin revealed that the C-terminal domain has an extended structure and is composed of one β-strand and six helical regions capable of binding to the FERM surface (Fehon, et al., 2010). Therefore, in the inactive/dormant state, ERM undergo intramolecular association, forming homo- or hetero-dimers of the proteins by bridging the FERM domain of ERM to the C-terminal ERM association domain (C-ERMAD) (Gary and Bretscher, 1995;Gary and Bretscher, 1993;Tsukita, et al., 1997) (Fig. 1.4B).

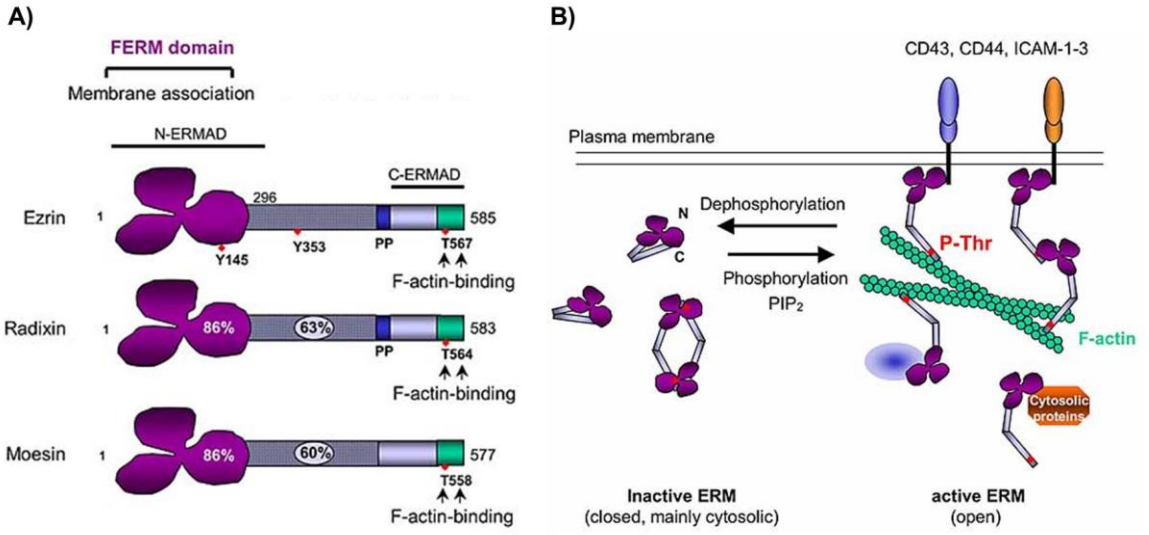


Figure 1.4. Structure of ERM family proteins and model for activation.
(A) Schematic depicting the structure of ERM, with percentage of homology of each protein to ezrin shown. The domains involved in intra- and inter-molecular interactions of ERM proteins, including N-ERMAD (N-terminal ERM association domain) and C-ERMAD (C-terminal ERM association domain) are also shown. The F-actin binding domain and the conserved C-terminal Thr sites for phosphorylation are also denoted. (B) ERM proteins exist in a 'dormant' state, in which proteins are in a folded conformation, and an 'active' state, in which the protein is unfolded and fully capable of interacting with membrane proteins and the cytoskeleton. ERM transition into the 'active' state requires binding of phosphoinositides to the FERM domain and by phosphorylation of the critical C-terminal Thr residues. Activated ERM participate in cytoskeleton-membrane linkage via direct association with transmembrane proteins, such as CD43, CD44 (hyaluronan receptor) and ICAM (intercellular adhesion molecule) 1-3, or indirectly through scaffolding proteins, including EBP50 (ERM-binding phosphoprotein) [Adapted and modified from (Charrin and Alcover, 2006)].

Transitioning ERM into an 'active state' involves conformational changes induced by binding of phosphoinositide's, such as phosphatidylinositol 4,5-bisphosphate (PIP_2) to the FERM domain, which renders the conserved Thr residues on C-ERMAD of ERM (T567 in ezrin, T564 in radixin and T558 in moesin) to be more accessible to phosphorylation (Matsui, et al., 1998;Nakamura, et al., 1999;Fievet, et al., 2004;Ben-

Aissa, et al., 2012). Hence, this mechanism not only unmasks binding's sites for membrane proteins on the FERM domain, but also those for F-actin on the C-ERMAD domain of ERM. This enhanced phosphorylation of T558 of moesin was originally shown to be important for activation of platelets (Nakamura, et al., 1999), and subsequently for all ERM, which reduces affinity of the C-ERMAD for the FERM domain and regulates their head-to-tail association (Matsui, et al., 1998).

Activated ERM proteins participate in cytoskeleton-membrane linkage by mechanisms involving the direct association with transmembrane proteins, including the hyaluronan receptor CD44, CD43 (leukosialin) and intercellular adhesion molecule 1-3, or indirectly through scaffolding proteins, such as the ERM-binding phosphoprotein (EBP50) (Fig. 1.4B). ERM phosphorylation/activation is evidently important for neuronal function (Lamb, et al., 1997;Paglini, et al., 1998;Haas, et al., 2007;Jeon, et al., 2010) and for activation of microglia during traumatic brain injury (Moon, et al., 2011)or in response to LPS (Kashimoto, et al., 2013). In addition, ERM phosphorylation has been shown to be important for regulation of MC activation (Sieghart, et al., 1978;Wells and Mann, 1983;Wang, et al., 1999;Correia, et al., 1996;Theoharides, et al., 2000;Olson, et al., 2009;Halova, et al., 2013;Staser, et al., 2013). Noteworthy, multiple tyrosine sites for phosphorylation on ezrin (Tyr145/Tyr353) have also been identified (Ramesh, 2004). Conversely, ERM inactivation can occur by dephosphorylation of the critical C-ERMAD Thr residue (Westphal, et al., 1999;Shi, 2009), while in MC phosphorylation on Ser56/74 and/or Thr66 residues within the FERM domain of moesin have been associated with inhibition of mediator release (Correia, et al., 1996;Wang, et al., 1999;Kempuraj, et al., 2005). Therefore, ERM are effectors of several intracellular signaling pathways.

ERM Proteins as Signal Transducers

ERM proteins function as signal transducers via direct or indirect interactions with the PI3K/AKT/mTOR pathway and serve as substrates for several kinases downstream of mTORC2 signaling (Tsukita and Yonemura, 1997;Bretscher, 1999;Bretscher, et al., 2002;McClatchey, 2014). ERM can interact with the upstream inhibitory mTOR protein TSC1/hamartin to modulate Rho signaling(Lamb, et al., 2000;Rosner, et al., 2008), while indirect association of ERM with PTEN, at the plasma membrane has recently been shown to be mediated by its scaffolding protein EBP50 (Zheng, et al., 2016a). In fact, PTEN the upstream inhibitory mTOR protein, and a phosphatase could regulate levels of PIP2 consequently activation of ERM. Moreover, ezrin has been shown to interact with PI3K and AKT to regulate epithelial cell survival (Gautreau, et al., 1999). Interaction of Akt and phosphorylated moesin was also shown to be required for neuronal growth in response to NGF (Jeon, et al., 2010).

At least three known kinases downstream of mTORC2 signaling that are implicated in cytoskeleton rearrangement (Sarbassov, et al., 2004;Saci, et al., 2011;Gulhati, et al., 2011;Oh and Jacinto, 2011), including the Rho family (Matsui, et al., 1998;Hall, 2012), protein kinase C (PKC) α and PKC isoform θ (Pietromonaco, et al., 1998;Bretscher, et al., 2002) have been shown to phosphorylate moesin or ezrin on the conserved Thr residue for activation of ERM. Interestingly, RhoA (Gulhati, et al., 2011) and Rac1 (Saci, et al., 2011) that have been shown to associate with mTORC2, regulating its kinase activity, have also been reported to regulate MC exocytosis (Brown, et al., 1998), suggesting that ERM activation is important for MC activation and mediator release.

1.6. Mast cells and Keratinocytes

Communication between MC and keratinocytes: Relevance to inflammation of the skin

MC are critical regulators of skin immunity for host defense (Galli, et al., 2005) and wound healing (Willenborg, et al., 2014) to maintain the regulation vascular permeability of skin-blood barrier and angiogenesis (Metcalfe, et al., 1997). MC reside in the upper dermal layer of the skin proximal to sensory nerve endings and blood vessels (Harvima and Nilsson, 2011). During chronic skin inflammation, as in psoriasis, the infiltration of MC (Harvima and Nilsson, 2011) and abnormal activation has been denoted (Maurer, et al., 2003;Castells, 2006;Harvima, et al., 2008;Theoharides, et al., 2004a;Sismanopoulos, et al., 2013). MC can be activated by various stimuli, such as cytokines, chemokines and neuropeptides to release multiple mediators with potent vasodilatory, inflammatory and pruritic properties (Theoharides and Cochrane, 2004), including histamine, IL-6, CXCL8, TNF and VEGF, leading to local vascular activation and subsequent immune cell recruitment (Vocanson, et al., 2009). Importantly, MC are the only immune cells that store preformed TNF (Zhang, et al., 2012a), which could stimulate keratinocytes (Sedger and McDermott, 2014;Grine, et al., 2015).

Keratinocytes that reside in the epidermal skin layer are a source of pro-inflammatory cytokines and growth factors, including stem cell factor (SCF) (Welker, et al., 1995), which is a ligand for the MC KIT receptor and essential for the growth, migration and survival of MC (Grabbe, et al., 1996;Enomoto, et al., 2011). Keratinocytes are considered fully immunocompetent and capable of IL-1β, IL-6, IL-8, TNF and VEGF

secretion, in response to immune and endocrine triggers (Parrado, et al., 2012;Canavese, et al., 2010;Slominski, et al., 2013).

Communication between MC and keratinocytes is important in the pathogenesis of psoriasis, a chronic, inflammatory and autoimmune skin condition, characterized by increased keratinocyte proliferation and a disease pathology that involves the presence of skin lesions, plaques, redness and itching (Ozdamar, et al., 1996;Harvima, et al., 2008;Nestle, et al., 2009;Eberle, et al., 2016). Activated keratinocytes could also produce IL-1β, which stimulates MC release of TNF that could act back on keratinocytes to release a number of other inflammatory mediators. MC-derived proteases stimulate PAR2 receptors on keratinocytes (Schechter, et al., 1998), while histamine induces expression of H4R and CXCL8/IL-8 in keratinocytes (Suwa, et al., 2014) and stimulates keratinocyte proliferation (Glatzer, et al., 2013). In addition, MC-derived histamine increases NGF production from keratinocytes (Groneberg, et al., 2005), which in turn stimulates histamine release from MC, generating a positive feedback-loop (Kanda and Watanabe, 2005) (Fig. 1.5).

Recent evidence further shows that MC release IL-17A (Suurmond, et al., 2011) and IL-33(Hsu, et al., 2010), which also stimulates keratinocyte activation (Kirkham, et al., 2014;Li, et al., 2015;Muromoto, et al., 2016). In addition, activated MC could induce T cell activation via TNF (Kempuraj, et al., 2008). In fact MC can function as immunomodulatory cells (Galli, et al., 2008a) and are important for the maturation of T_h17 cells, which are recognized as key cells in autoimmune disorders by releasing IL-17A and IL-22 (O'Connor, Jr., et al., 2010). MC-derived TNF can stimulate IL-33 release

from keratinocytes (Balato, et al., 2012). Moreover, our studies has shown that IL-33 augments SP-stimulated VEGF release from human mast cells; IL-33 gene expression is also increased in lesional skin of Ps patients (Theoharides, et al., 2010) and elevated serum IL-33 levels have also been reported (Mitsui, et al., 2015). Hence, cross-talk between MC and keratinocytes may exacerbate inflammatory processes in psoriasis (Fig. 1.5).

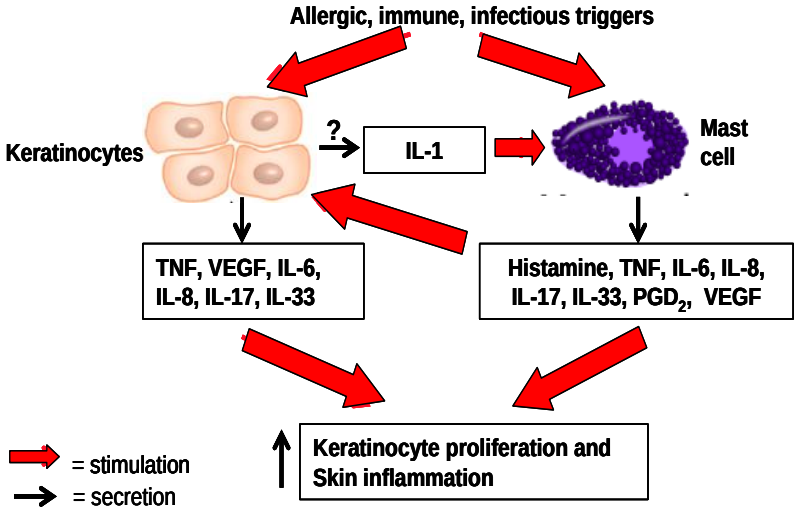


Figure 1.5. A depiction of cross-talk between MC and keratinocytes involved in chronic skin inflammation and psoriasis. Multiple stimuli, including NT or SP and TNF could stimulate synergistic activation of MC and keratinocyte. Activated keratinocytes release IL-1β, which stimulates MC to secrete histamine and TNF, IL-17A and IL-33. These cytokines could further stimulate MC and keratinocyte release of CXCL8 and VEGF, and keratinocyte hyper-proliferation, contributing to inflammation of the skin and psoriasis [Figure adapted from Theoharides laboratory (unpublished)].

In addition, the increased levels of neuropeptides, especially CRH (Vasiadi, et al., 2012), NT(Vasiadi, et al., 2012a) and SP (Remröd, et al., 2007) have been reported in psoriasis. These peptides could stimulate activation of keratinocytes (Dallos, et al.,

2006;Shi, et al., 2013;Shi, et al., 2011) and MC (Singh, et al., 1999;Donelan, et al., 2006;Kulka, et al., 2007;Theoharides, et al., 2010). Hence, neuroimmunoendocrine interactions involving neuropeptides, keratinocytes and MC could be important for the pathophysiology of psoriasis (Scholzen, et al., 1998;Paus, et al., 2006;Theoharides, et al., 2004b;Theoharides, et al., 2016a).

Currently Available Treatments for Psoriasis

The prevalence of psoriasis in the US is ~3 % (Helmick, et al., 2014), and is considered a major public health problem due to ineffective long-term treatments that resulted in the annual economic burden amounting to $11 billion in 2013 (Vanderpuye-Org, et al., 2015;Brezinski, et al., 2015). Recent studies on the pathogenesis of psoriasis have revealed a genetic predisposition (Kaffenberger, et al., 2014;Tsoi, et al., 2012) and the role of immune dysfunction (Li, et al., 2016b), as a consequence of an imbalance in polarized T-helper subsets (T_h1-T_h2-T_h17)(Nestle, et al., 2009;Eberle, et al., 2016) and activation of keratinocytes (Albanesi, et al., 2007;Harvima, et al., 2008;Eberle, et al., 2016), skin-resident and infiltrating MC (Toruniowa and Jablonska, 1988;Paus, et al., 2006;Harvima, et al., 2008;Metz and Maurer, 2009;Theoharides, et al., 2010) and other immune cells, including dendritic cells and macrophages (Deng, et al., 2016).

Thus, the increased levels of the pro-inflammatory cytokines TNF (Olivieri, et al., 2009;Sedger and McDermott, 2014;Grine, et al., 2015), IFNγ, IL-1β (Nickoloff, et al., 2007), IL-6(Hunter and Jones, 2015), IL-17A (Kirkham, et al., 2014), IL-22, IL-23 (Coimbra, et al., 2012;Raychaudhuri, et al., 1998;Suzuki, et al., 2014) and IL-33 (Theoharides, et al., 2010;Balato, et al., 2012), as well as VEGF (Marina, et al., 2015)are all implicated in psoriasis. Moreover, the elevated levels of the peptides CRH (Vasiadi, et

al., 2012), NT (Vasiadi, et al., 2012a) and SP (Remröd, et al., 2007), could activate keratinocytes (Dallos, et al., 2006;Shi, et al., 2013;Shi, et al., 2011) and MC (Singh, et al., 1999;Donelan, et al., 2006;Kulka, et al., 2007;Theoharides, et al., 2010). Hence, the interactions among neuropeptides, keratinocytes and MC could be important for the pathophysiology of psoriasis (Scholzen, et al., 1998;Paus, et al., 2006;Theoharides, et al., 2004a;Theoharides, et al., 2016b).

The conventional treatments for the management of mild psoriasis include the topical application of glucocorticosteroids and Vitamin D analogs, both of which regulate epidermal keratinocyte immune responses (Victor, et al., 2003;Nestle, et al., 2009). Moderate psoriasis is typically treated with a combination of these and phototherapy, such as ultraviolet A/B to inhibit epidermal keratinocyte proliferation. Severe psoriasis, resistant to topical drugs or phototherapy, are usually treated with a combination of retinoids with either methotrexate or cyclosporine, which are immunosuppressant drugs. Newer and more costly biologics, among which are inhibitors for TNF (etanercept, adalimumab, infliximab) (Kotsovilis and Andreakos, 2014;Cohen, et al., 2015), IL-12 and IL-23 (Gomez-Garcia, et al., 2016) (ustekinumab) and 1L-17A (secukinumab) (Ryoo, et al., 2016) signaling pathways are available and soomewhat effective, but are associated with the increased risk of infections and cardiovascular events (Winterfield, et al., 2005;Kaffenberger, et al., 2014;Deng, et al., 2016) (Table 1.2.).

TABLE 1.2. Summary of anti-psoriatic therapies

Drug	Mechanism of Action	Efficacy	Adverse side-effects and common deficiency
Glucocorticosteroids	Inhibition of inflammatory reactions, DNA synthesis, vasoconstriction and immunosuppression	++++	Skin atrophy and loss of efficacy with rebound effects; only treat mild disease
Vitamin D analogues	Inhibits epidermal keratinocyte hyper-proliferation via blockade ofPI3K/mTOR (Mitra A, et al., 2013)	+++	Mild discomfort Fail to have rapid on-set of activation
Phototherapy (UVB, UVA)	Inhibits epidermal keratinocyte hyper-proliferation via blockade ofPI3K/mTOR; Shifts Th1 to Th2 type cytokines	+++	Skin premature aging; skin cancer
Methotrexate	Immunosuppression	+ to ++	Liver fibrosis; cirrhosis
Cyclosporin	Immunosuppression	++ to +++	Hypertension; malignancies; infections
Etanercept Type: Fusion protein human TNFRp75 with Fc of IgG1	Blocking TNF pathway	+ to +++	Infections; malignancies
Infliximab Type: Chimeric mAb of human & murine sequences	Blocking TNF pathway	+++ to ++++	Infections
Adalimumab Type: Fully human mAb	Blocking TNF pathway	+++	Infections; lupus-like syndrome; malignancies
Ustekinumab Type: Fully human mAb	Blocking IL-12/IL-23 signaling (Gómez-Garcia, F et al., 2016)	+++	Infections
Secukinumab Type: Fully human mAb	Blocking IL-17A signaling (Ryoo J, et al., 2016)	+++ to ++++	Respiratory infection; migraines

Global assessment: six levels for estimating efficacy
Poor---------- Good
-+-/-+ ++ +++ ++++
[Adapted and modified from (Winterfield L, et al., 2005;Kaffenberger B, et al., 2014; Deng Y, et al., 2016)]

1.7. Recent Therapeutic Avenues for Inflammation-related Diseases

Inhibitors of mTOR signaling as Potential Treatments for ASD & Psoriasis

The PI3K/AKT/mTOR signaling cascade which regulates cellular metabolism, proliferation and survival is frequently dsyregulated in a variety of cancers (Laplante and Sabatini, 2012), and has recently emerged as a clinically relevant target for neurodevelopmental disorders, including ASD (Sahin, 2012;Ehninger and Silva, 2011;Lipton and Sahin, 2014;Enriquez-Barreto and Morales, 2016) and psoriasis (Jegasothy, et al., 1992;Ormerod, et al., 2005;Frigerio, et al., 2007;Huang, et al., 2014;Eberle, et al., 2016). Interestinglly, inhibitors of mTOR signaling have also been shown to suppress activation of the pro-inflmmatory M1 microglia phenotype (Tateda, et al., 2016;Li, et al., 2016a), and the activation of MC (Kim, et al., 2008b;Smrz, et al., 2011), and keratinocytes (Mitra, et al., 2012;Zhang and Ma, 2014;DeTemple, et al., 2016).

Rapamycin (sirolimus) has had diverse clinical applications as an anti-fungal, immunosuppressant and anti-cancer drug (Sehgal, 2003;Paghdal and Schwartz, 2007) has been tried for the treatment of psoriasis (Ormerod, et al., 2005), and more recently for treatment of tuberous sclerosis (TSC1/2) and ASD (Ehninger and Silva, 2011). Although structurally similar to cyslosporine and tacrolimus, it does not inhibit the clamodulin-calcium-dependent phosphatase required for nuclear factor of activation T-cell to block transcription of interleukins/cytokines (i.e. a calcineurin inhibitor) (Paghdal and Schwartz, 2007). Instead, rapamycin interacts with a family of intracellular binding

proteins, the most relevant being FKBP12, which blocks mTOR activation (Paghdal and Schwartz, 2007).

Sirolimus is poorly absorbed orally with a bioavailability of 15 % and half-life is ~62 h, with maximal blood concentrations reached at ~2 h (Paghdal and Schwartz, 2007) . Hence, analogs of sirolimus, such as everolimus have been created to enhance the pharmokinetic and phamacodynamic profiles, as well as solubility. Everlimous, also immunosuppresive has been tried for the treatment of TSC1/2, where it improved seizures and behaivoral deficits (Hwang, et al., 2016) and is currently being tried for psoriasis (Frigerio, et al., 2007;Wei and Lai, 2015). However, several cutaneous adverse effects have been associated with the clinical use of these allosteric mTOR inhibitors (Macdonald, et al., 2015) and patients are prone to infections due to the immunosuppresive properties of the drugs. Acute renal failure has also been observed in some TSC1/2 patients treated with everlimous (Curatolo, et al., 2016). Thus, there is a cruicial need for the development of new clinical inhibitors for PI3K/mTOR signaling. Intriguingly, numerous flavonoids, natural phytochemicals have been reported to inhibit the PI3K/AKT signaling cascade in inflammatory skin-disorders (Leo and Sivamani, 2014) and inflammation of the brain (Leyva-Lopez, et al., 2016), without adverse effects.

Flavonoids as Potential Therapy for Inflammatory Disorders

Flavonoids are naturally-occurring phytochemicals in green plants and seeds (Middleton, et al., 2000;Taylor and Grotewold, 2005), with many beneficial actions occurring in a wide range of structural diversity (Agati, et al., 2012) and beneficial actions. The six major subclasses of flavonoids include the flavones (e.g., apigenin, luteolin), flavonols (e.g., quercetin, myricetin), flavanones (e.g., naringenin,

hesperidin), catechins or flavanols (e.g., epicatechin, gallocatechin), anthocyanidins (e.g., cyanidin, pelargonidin), and isoflavones (e.g., genistein, daidzein) (Ross and Kasum, 2002). Flavonoids possess favorable anti-oxidant, anti-inflammatory, anti-allergic and anti-proliferative properties for the treatment and prevention of diabetes (Babu, et al., 2013), cardiovascular (Basu, et al., 2016), cancer (Middleton, Jr. and Kandaswami, 1993;Middleton, et al., 2000) and neurodegenerative diseases (Jones, et al., 2012). Importantly, flavonoids are modulators of inflammatory cytokines (Leyva-Lopez, et al., 2016) and chemokines by targeting signaling pathways to suppress inflammatory processes (Tunon, et al., 2009).

Luteolin (3',4',5,7-tetrahydroxyflavone) inhibits MC-mediated allergic inflammation (Kritas, et al., 2013), LPS-stimulated pro-inflammatory responses in murine microglia (Jang, et al., 2008) and keratinocyte activation (Weng, et al., 2014) via mechanisms involving suppression of the nuclear factor kappa B (NF-κB) activation and gene expression of pro-inflammatory mediators (Seelinger, et al., 2008). Luteolin, not only inhibits MC degranulation (Kimata, et al., 2000a) and cytokine release (Asadi and Theoharides, 2012), but also MC-induced T cell activation (Theoharides, et al., 2007a;Kempuraj, et al., 2008). Luteolin is also considered as a neuroprotective agent (Dajas, et al., 2003;Nabavi, et al., 2015) .

In fact, luteolin triggers global changes in the murine microglial transcriptome, resulting in a neuroprotective phenotype (Dirscherl, et al., 2010) and opposes MIA activation, reversing ASD-like behavior in mice (Parker-Athill, et al., 2009). Our laboratory reported that a luteolin-containing dietary formulation had beneficial effects in reducing ASD symptoms (Theoharides T.C., et al., 2012a;Taliou, et al., 2013), and could

even protect against memory loss (Theoharides, et al., 2015b). Therapeutically, luteolin is generally safe (Formica and Regelson, 1995;Kawanishi, et al., 2005;Harwood, et al., 2007;Li, et al., 2010), and can even protect against drug-induced hepatotoxicity (Domitrovic, et al., 2009) and nephrotoxicity(Domitrovic, et al., 2013).

Structural analogs of luteolin, such as the 7,8-dihydroxyflavone, a pro-neurotrophic compound, which mimics BDNF and is a tyrosine kinase receptor agonist has also shown promising therapeutic efficacy for neurodevelopmental disorders (Du and Hill, 2015;Liu, et al., 2016). Interestingly, 7,8-dihydroxyflavone was reported to attenuate release of IL-1β and TNF from murine microglia by inhibiting NF-κB activation (Park, et al., 2014). Luteolin and its structural analogs can also directly block activation of the toll-like receptors (Zhao, et al., 2011), suggesting another mechanism for flavonoid mediated inhibition of LPS-stimulated pro-inflammatory responses.

Recent studies in our laboratory have focused on another structural analog, 3',4',5,7-tetramethoxyluteolin (methoxyluteolin), which is more lipid soluble and more potently than luteolin, inhibits pro-inflammatory cytokine and chemokine release from cultured human MC (Weng, et al., 2015). This mechanism of action of methoxyluteolin was speculated to involve blockade of intracellular calcium influx and/or induction NF-κB. Interestingly, the PI3K/mTOR pathway is upstream of NF-κB and could regulate activation of the latter transcription factor (Dan, et al., 2008;Laplante and Sabatini, 2013).

Evidence also indicates that mTOR activation promotes the pro-inflammatory M1 phenotype of microglia (Li, et al., 2016a) and the proliferation and FcεRI-mediated allergic responses of MC (Kim, et al., 2008a;Smrz, et al., 2011). In addition, the PI3K/mTOR pathway also regulates proliferation of epidermal keratinocytes (Mitra, et

al., 2012) and keratinocyte activation (Zhang and Ma, 2014;Datta, et al., 2013;Raychaudhuri and Raychaudhuri, 2014). Moreover, in human oral keratinocytes stimulation via the toll-like receptor activates mTOR and NF-κB, resulting in increased cytokine production (Zhao, et al., 2010). Thus, it is important to determine whether luteolin and methoxyluteolin can inhibit PI3K/mTOR activation in stimulated microglia, MC and keratinocytes for beneficial treatment.

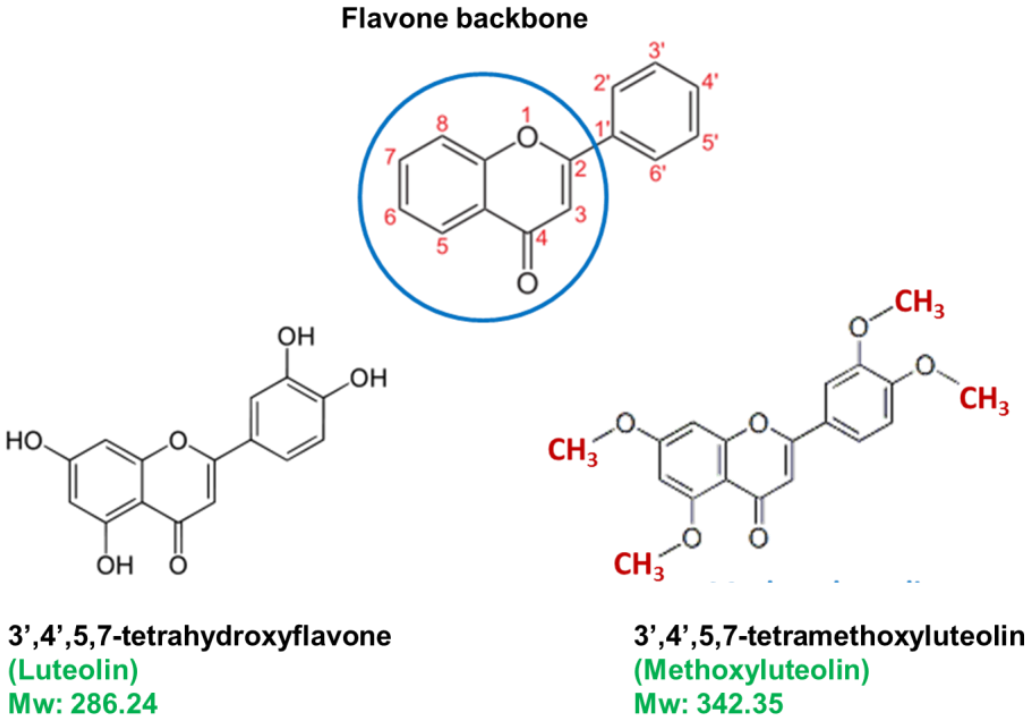


Figure 1.6. Structure of the flavonoids luteolin and methoxyluteolin. The structure of the flavone back-bone, with its numbering scheme is shown (top), and the differences in the chemical structures of the flavonoids luteolin (3',4',5,7-tetrahydroxyflavone) and its structural analog 3',4',5,7-tetramethoxyluteolin (methoxyluteolin) are also shown. The four additional methyl groups on methoxyluteolin, as compared to luteolin increases its hydrophobic character, and hence its solubility, absorption and metabolic stability. [Adapted from Theoharides laboratory (unpublished)]

1.8. Dissertation Summary

Hypothesis

We hypothesized that stimulation of human microglia, MC and keratinocytes resulting in the increased pro-inflammatory cytokine/chemokine gene expression and protein release, will involve activation of mTOR, which will be the molecular target for inhibition by the novel flavonoid methoxyluteolin to effectively attenuate these aberrant cellular responses.

Objectives

Previously, our laboratory had reported the elevated serum levels of the peptide neurotensin (NT) in children with Autism Spectrum Disorders (ASD) (Angelidou, et al., 2010;Tsilioni, et al., 2014). In chapter 2, our studies investigated whether NT could stimulate the activation of human microglia using cultured primary microglia, obtained from human brain tissues and the immortalized cell line of human microglia-SV40. We determined whether NT could increase the gene expression and release of pro-inflammatory cytokine and the chemokines from human microglia, and identified the specific NT receptor implicated in mediating these responses. We determined that the soluble protein of this NT receptor is increased in the serum from children with ASD, as compared to normal controls. Studies have also reported that mutations in upstream negative regulatory proteins, which in effect will increase activation of mTOR, are associated with the risk of some ASD (Fig. 1.7A).

Hence, in Chapter 2 we also investigated the importance of PI3K/mTOR signaling cascade in the activation of human microglia in response to NT, by using specific

pharmacological inhibitors and evaluated the inhibitory effects of the flavonoids luteolin and methoxyluteolin on mTOR activation and microglial responses (Fig.1.7B). Our findings for the first-time link a physiologically relevant trigger with the pathological findings of microglia activation and inflammation found in brains of patients with ASD, as well as show that inhibition PI3K/mTOR pathway using methoxyluteolin could be translated into an effective treatment of ASD.

Previously, our laboratory has reported that in addition to the allergic stimulation of MC, the peptides NT (Alysandratos, et al., 2012;Vasiadi, et al., 2012a), and substance P (SP) (Asadi, et al., 2012;Weng, et al., 2015) stimulate MC degranulation and release of newly-synthesized pro-inflammatory cytokines and chemokines. We had also reported the elevated circulating levels of NT in patients with inflammatory skin disorders, such as psoriasis (Vasiadi, et al., 2012a), and in children with ASD (Angelidou, et al., 2010;Tsilioni, et al., 2014), while increased levels of SP have been associated with psoriasis (Remröd, et al., 2007).In chapter 3, our studies investigated the activation of the PI3K/mTOR signaling pathway in human MC in response to stimulation by NT and SP using the immortalized LAD2 MC line. We determined whether the NT- and SP-stimulated pro-inflammatory mediator gene expression and release from human MC could be attenuated by the pharmacological inhibition of PI3K/mTOR signaling (Fig. 1.7), and if mTOR is a molecular target of inhibition by luteolin and methoxyluteolin. Our findings indicate that mTOR activation is critical for the neuropeptide-induced pro-inflammatory gene expression and release from human MC, while its blockade by methoxyluteolin provides a treatment option for inflammatory skin disorders.

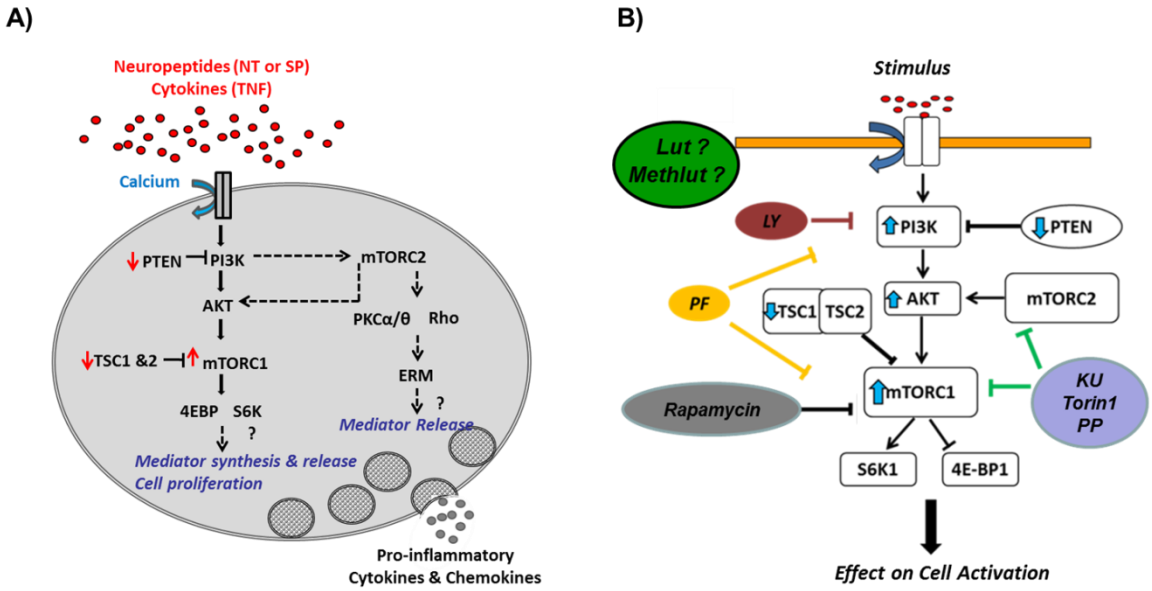


Figure 1.7. Schematic showing the hypothesized involvement of mTOR in aberrant cell activation in response to physiological stimuli and blockade via pharmacological inhibitors and flavonoids. (A) Cellular influx of calcium activates the PI3K/AKT/mTOR signaling cascade, which typically regulates cellular homeostasis. Mutations associated with the risk of ASD which include PTEN or TSC1/2 decrease the respective protein levels (red arrows), removing the inhibitory regulation of mTORC1. The expression of mTORC1 signaling proteins is also elevated in lesional psoriatic skin (red arrow). mTOR (pSer2448) activates S6K (p70S6KThr389) and inhibits 4EBP (pThr36/47) to regulate mediator protein translation and cell proliferation. mTORC2 activates AKT (pSer473) to regulate mTORC1 signaling, and mediates cytoskeletal organization via activation of the PKC α or θ isoforms, and/or the Rho family of GTPases, upstream of the ERM cytoskeletal proteins. Activation of ERM promotes cross-linking of F-actin cytoskeleton to the plasma membrane to promote cell secretion. (B) Involvement of PI3K/mTOR signaling in mediating cellular activation will be assessed using specific pharmacological ATP-competitive inhibitors of PI3K (LY294002, LY), dual PI3K/mTOR (PF-04691502, PF) and the dual mTORC1/mTORC2 (KU0063794, KU; Torin1; PP242, PP), as well as the allosteric inhibitor of mTORC1 (Rapamycin, Rap), a bacterial macrolide. The mechanism of action of the flavonoids luteolin (Lut) and its structural analog methoxyluteolin (Methlut) for inhibition of PI3K/mTOR signaling in activated microglia, MC and keratinocytes will also be studied

In chapter 4, we investigated the importance of ERM and its phosphorylation (activation) for the regulation of human MC mediator release in response to allergic or neuropeptide stimuli by using the siRNA knockdown approach to downregulate ezrin and

moesin in LAD2 MC. We determined the predominant ERM implicated in the regulation of human MC degranulation and in the release of newly-synthesized pro-inflammatory cytokines and chemokines after IgE/anti-IgE or SP stimulation.We also investigated whether blockade of the PI3K/mTOR pathway using specific inhibitors (Fig 1.7), and the flavonoids luteolin and methoxyluteolin. Our findings indicate that both ezrin and moesin are required for the regulation of human MC mediator release, while activation of ERM proteins, specifically moesin in MC is dependent on PI3K/mTOR signaling. Methoxyluteolin inhibits activation of moesin, indicating a novel molecular target of inhibition and provides a treatment option for aberrant MC activation in inflammatory disorders.

In chapter 5, our studies investigated the activation of the PI3K/mTOR signaling pathway in human keratinocytes in response to stimulation by tumor necrosis factor (TNF) cytokine using normal human epidermal keratinocytes (NHEKs) and the immortalized human keratinocyte cell line (HaCaT). We determined whether TNF-stimulated pro-inflammatory mediator gene expression and release from human keratinocytes could be attenuated by the pharmacological inhibition of PI3K/mTOR signaling (Fig. 1.7), and if mTOR is a molecular target of inhibition for the flavonoids luteolin and methoxyluteolin. Our findings indicate that mTOR activation is critical for the TNF-induced pro-inflammatory gene expression and release from human keratinocytes, while its blockade by methoxyluteolin provides a treatment option for inflammatory skin disorders, such as psoriasis.

Chapter 2

Neurotensin (NT) Stimulation of Human Microglia via NTR3/Sortilin Receptor Elevated in Autism involves mTOR activation and is Inhibitable by Methoxyluteolin

2.1. Introduction

Autism Spectrum Disorders (ASD) are neurodevelopmental disorders (Lai, et al., 2014;Volkmar and McPartland, 2014). The prevalence of ASD is now estimated to be 1 in 45 children (Zablotsky B and et al., 2015). Unfortunately, there is still no distinct pathogenesis (Willsey and State MW, 2015) even though a number of neuropathological defects have been reported in the brains of children with infantile autism (Kemper and Bauman, 2002). Microglia, the highly plastic resident immune cells of the brain (Paolicelli, et al., 2011;Shemer, et al., 2015), have been shown to be activated in the brains of patients with ASD (Vargas, et al., 2005;Morgan, et al., 2010;Rodriguez and Kern, 2011;Morgan, et al., 2014). Microglia activation and proliferation could lead to focal inflammation of the brain and "choking" of normal synaptic connectivity (Suzuki, et al., 2013;Edmonson, et al., 2014). Microglia express membrane receptors for several neuropeptides, allowing them to communicate with neurons, astrocytes (Pannell, et al., 2014) and mast cells (Skaper, et al., 2012), known to be involved in allergic and inflammatory processes (Theoharides, et al., 2015d).

Various stimuli, such as the bacterial lipopolysaccharide (LPS) (Jang, et al., 2008;Pannell, et al., 2014), have been shown to switch microglia into the M1 phenotype, denoted by the release of pro-inflammatory cytokines, interleukin (IL)-1beta, IL-6 and tumor necrosis factor (TNF) (Li, et al., 2009), as well as the chemokines (C-C motif) ligand 2 (CCL2) and CCL5 (Vargas, et al., 2005;Ashwood, et al., 2011), also found to be increased in brains of deceased patients with ASD. Immune dysfunction (Zimmerman, et al., 2005;Li, et al., 2009;Estes and McAllister, 2015;Young, et al., 2016) and inflammation of the brain (Hagberg, et al., 2012;Theoharides, et al., 2013;Theoharides, et

al., 2015c) are now invoked in the pathogenesis of ASD. However, the stimuli, which promote these inflammatory processes in the brain are presently unknown.

Our laboratory had reported increased serum levels of the peptide neurotensin (NT), but not substance P or β-endorphin (Angelidou, et al., 2010), in children with ASD (Angelidou, et al., 2010;Tsilioni, et al., 2014). NT is found in the brain (Carraway and Leeman, 1973;Dobner, et al., 1987) and is primarily secreted from neurons (Dobner, et al., 1987) and astrocytes (Vincent, et al., 1994). NT responses are mediated through three receptors: NTR1 (Navarro, et al., 2001) and NTR2 (Chalon, et al., 1996;Mazella, et al., 1996), which belong to the G protein-coupled seven-transmembrane receptor family (Vincent, et al., 1999), and NTR3 also known as sortilin (Petersen, et al., 1997). NTR3/sortilin is a type I sorting protein, part of the Vps10p domain single transmembrane receptor family (Navarro, et al., 2001), a multifaceted receptor mainly expressed in the CNS during embryonic development (Hermans-Borgmeyer, et al., 1999).

NTR3/sortilin has been shown to be expressed in murine microglia through which NT stimulates IL-1β, CCL2 and TNF gene expression (Martin, et al., 2005). However, rodent microglia have major biochemical and pharmacological differences, when compared to primary human microglia (Smith and Dragunow, 2014). Moreover, animal models do not reflect human inflammatory processes (Seok, et al., 2013). A subset (1-5%) of ASD cases have gene mutations in regulatory proteins, upstream of the signaling complexes termed the mammalian target of rapamycin (mTOR) (Willsey and State MW, 2015;Lee, 2015). These mutations in mice lead to a behavioral phenotype resembling autism (Kwon, et al., 2006) and targeting the mTOR pathway has been shown to reverse

autism-like behavior (Wang and Doering, 2013;Huber, et al., 2015). The phosphoinositide 3-kinase (PI3K)/AKT/mTOR signaling pathway also regulates the activation of both microglia (Dello, et al., 2009) and mast cells (Kim, et al., 2008a), which may cross-talk to exacerbate inflammation of the brain (Skaper, et al., 2012).

There are no clinically available drugs addressing the core symptoms of ASD. The natural flavonoid luteolin has potent anti-oxidant and anti-inflammatory properties (Middleton, et al., 2000). It also inhibits activation of microglia (Jang, et al., 2008;Dirscherl, et al., 2010;Kao, et al., 2011;Zhu, et al., 2014). Luteolin also reverses autism-like behavior in mice (Parker-Athill, et al., 2009). Two clinical studies further reported that a luteolin-containing dietary formulation significantly improved attention and sociability in children with ASD (Theoharides T.C., et al., 2012a;Taliou, et al., 2013). Its structural analog, methoxyluteolin (3',4',5,7-tetramethoxyflavone) is a more potent mast cell inhibitor (Weng, et al., 2015), and more metabolically stable (Walle, 2007), hence more likely to reach therapeutic levels in the brain.

Here we extend our previous finding of elevated serum NT levels in children with ASD by investigating whether NT stimulates the activation of human microglia, the specific NT receptor involved and pathways that can be targeted for inhibition of these processes by methoxyluteolin.

2.2. Materials & Methods

Materials

The various stimuli, such as LPS (*E.coli* 0111:B4), NT (N6383) and NT fragment 8-13 acetate (N5266), and NT receptor (R) antagonists SR48692 (selective for NTR1) and SR 142948A (nonselective for NTR1 or NTR2) were obtained from Sigma-Aldrich (St Louis, MO). The dual PI3K/mTOR inhibitor PF 04691502 and the mTOR inhibitors rapamycin and KU-0063794 were purchased from TOCRIS (Biosciences, Bristol, UK). The flavonoids luteolin and methoxyluteolin were obtained from Pharmascience Nutrients (Clear Water, FL). Silencer® Ambion Select siRNAs targeting human NTR3/sortilin (#1: s224558 & #2:s12404) and scramble siRNA non-targeting controls (#1 & #2) were obtained from Life Technologies (Grand Island, NY). RNeasy Mini (Qiagen Inc., Valencia, CA) and iScript cDNA synthesis kits (BioRad, Hercules, CA) were purchased. Taqman gene expression primers/assays for NTR1 (Hs00901551_m1), NTR2 (Hs00892563_m1), NTR3/SORT1 (Hs00361760_m1), NTR4/SORL1 (Hs00268342_m1), IL-1β (Hs01555410_m1), CXCL8 (Hs00174103_m1), CCL2 (Hs00234140_m1), CCL5 (Hs00174575_m1) and GAPDH endogenous control (4310884E) were purchased from Applied Biosystems (Foster City, CA). The Human Cytokine Array Panel A to simultanoeusly detect various human cytokines/chemokines and ELISA kits for human IL-1β (DY201), CXCL8/IL-8 (DY208), CCL2/MCP-1 (DY279) and CCL5/RANTES (DY278) were purchased from R&D Systems (Minneapolis, MN). The human SORT1 ELISA kit for detection of serum NTR3/sortilin was obtained from LifeSpan BioSciences, Inc. (Seattle, WA).

The Human Phospho-Kinase Array to detect multiple phosphorylated (p) kinases and specific phospho-ELISA kits for pAKT Ser473 (DYC88), pmTOR Ser2448 (DYC1665) and pp70S6K Thr389 (DYC896) were purchased from R&D Systems. The monoclonal (mAb) or polyclonal antibodies (pAb) for Western blot analysis and immunofluorescence were purchased and include the following: Mouse mAb NTR1(G-9):sc-393205; Mouse mAb NTR3(G-11): sc-376561; Goat pAb NTR3 C20: sc-25055; Goat pAb Iba1 (C-20): sc-28530; Rabbit pAb TREM-2 (H-160): sc-48764; Integrin αM (2LPM19c): sc-20050 (Santa Cruz Biotechnology Inc., Dallas, Texas); Rabbit pAb Anti-Sortilin (ab16640); Goat pAb Anti-Iba1 (ab5076) (abcam, Cambridge, MA); Rabbit pAb anti-NTR2 (EMD Millipore Corp., Temecula, CA); Rabbit mAb anti-SORL1/NTR4; Rabbit pAb SORT1/NTR3 HPA006889 (Sigma Aldrich, St Louis, MO); Rabbit mAb for Akt (pan-11E7); pAktSer473 (D9E); pAktThr308 (C31E5E) mTOR (7C10), pmTORSer2448 (D9C2), pmTORSer2481, mTORC1 substrates p70S6K (49D7), pp70SK Thr389 (108D2), 4EBP1 (53H11) and p4EBP1Thr37/46 (236B4), as well as the loading control β-actin (D6A8) were purchased from Cell Signaling Technology (Beverly, MA); CD11b/Integrin αM, Iba1 and NTR3/sortilin (Abcam, Cambridge, MA). For immunofluorescence microscopy the following secondary Ab and nuclei stain were purchased: Goat anti-rabbit IgG-FITC: sc-2012; Goat anti-mouse FITC: sc-2010; donkey anti-goat IgG-TR: sc-2783; donkey anti-goat IgG-FITC: sc-2024 (Santa Cruz Biotechnology Inc); anti-rabbit Alexa 488; anti-goat Alexa 594; DAPI or Hoechst (Life Technologies). MTT based in vitro toxicology assay kit was obtained from Sigma Aldrich.

Methods

Human Microglia Cell Culture

The immortalized human microglia-SV40 cell line derived from primary human microglia was purchased from Applied Biological Materials Inc. (ABM Inc., Richmond, BC, Canada) and cultured in Prigrow III medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in type I collagen-coated T25-flasks (ABM Inc.). Microglia-SV40 maintained specific phenotype and proliferation rates for over 10 passages, during which all experiments were performed using multiple microglia thaws and sub-cultured cells. Experiments were carried out in type-I collagen coated plates or four-well chamber slides (BD PureCoat ECM Mimetic Cultureware Collagen I peptide plates, Becton Dickinson, Bedford, MA). Primary human microglia (HM) isolated from human brain tissue were purchased from ScienCell Research Laboratories (Carlsbad, CA) and cultured in Microglia Medium supplemented with 5 % FBS, 1 % penicillin/streptomycin and 5 % microglia growth supplement in poly-L-lysine-coated T-25 flasks (ScienCell) or Chamber Slide Lab-Tek II CC2 chamber slides (Thermo Fisher Sci., Hudson, New Hampshire). Primary HM were not sub-cultured and were used within 7 d after cultures were initiated. Cell viability was determined by trypan blue (0.4 %) exclusion.

Human Microglia Treatments

Human microglia were stimulated by NT or LPS, and/or pre-treated with NTR1 selective non-peptide antagonist SR48692 or nonselective antagonist NTR1/2 (10-1000 nM) (Sigma Aldrich, St. Louis, MO) or the dual PI3K/mTOR inhibitor PF 04691502,

(PF, 10 nM-1000 nM, 2 h) (TOCRIS Biosciences, Bristol, UK) or mTOR inhibitors rapamycin (Rap, 10 nM-1000 nM,24 h) or KU-0063794 (KU, 10-1000 nM, 2 h), and the flavonoids (luteolin or methoxyluteolin, 0.1-10 μM; 2, 12 or 24 h, PharmaScience Nutrients, Clear Water, FL). All inhibitors were dissolved in water or DMSO (final concentration of <0.1 %). For siRNA knockdown experiments, two different predesigned and validated Silencer® Select siRNAs targeting human NTR3/sortilin and control siRNAs were purchased from Life Technologies (Grand Island, NY). siRNA (10-100 nM) transfection was carried out using Lipofectamine™ RNAiMAX in Opti-MEM® reduced serum and antibiotic-free medium (Life Technologies) for 48 h prior to evaluation of gene knockdown quantitative real-time PCR (qRT-PCR) and protein analysis by Western blot analysis.

Detection of NT Receptor Expression

The presence of gene expression of NTR3/sortilin was determined by qRT-PCR using specific primers and antibodies to distinguish among the three known NT receptor subtypes NTR1, NTR2 and NTR3/sortilin. Receptor gene expression of NTR1, NTR2 and NTR3/sortilin were measured by qRT-PCR using Taqman gene expression assays and validated primers (Applied Biosystems, Grand Island, NY). Total RNA from control and NT-stimulated (NT full-length of the active fragment residues 8-13) microglia (2.5×10^5 cells per 6-well type I collagen or poly-L-lysine-coated plates (Becton Dickinson) for 24 h before stimulation with NT (1-1000 nM) or LPS (10-1000 ng/mL) (Sigma-Aldrich) was carried out. coated-plates) was isolated after 6, 12 or 24 h using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription (RT) was performed with 300 ng of total RNA using the iScript

cDNA synthesis kit (Bio-Rad, Hercules, CA). For qRT-PCR, samples were run at 45 cycles using Applied Biosystems 7300 Real-Time PCR System. Relative mRNA abundance was determined from standard curves run with each experiment. Gene expression was normalized to GAPDH used as an endogenous control. The gene expression of receptors of NT (NTR1, NTR2 and NTR3) and the cell-type specific antigens for the microglial lineage, CD11b and CD86, were also determined.

Protein levels of NTR1, NTR2 and NTR3/sortilin were determined by Western blot analysis on cellular lysates harvested from microglia (1×10^6 cells) stimulated with NT (1-100 nM) for 24 h or 48 h. The membranes were probed with the following primary antibodies: NTR1 G9 (Santa Cruz Biotech. Inc., Dallas, TX), NTR2 (EMD Millipore Corporation, Temecula, CA), SORT1 (Sigma Aldrich, St Louis, MO), NTR3 C20, TREM-2 and Iba1 (Santa Cruz Biotech. Inc.), and β-actin for the loading control (Cell Signaling Technology/CST, Danvers, MA). All proteins were visualized with horseradish-peroxidase conjugated secondary antibodies and then by enhanced SuperSignal West Pico chemiluminescence (Fisher Sci., Pittsburgh, PA).

Cellular localization studies on NTR3/sortilin in basal and NT-stimulated microglia were done using confocal microscopy. Microglia (5×10^3 cells/four-well chamber coated slide) were stimulated with NT (10 or 100 nM NT; 12 h or 24 h). Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min on ice, and/or permeabilized using a solution of 0.1% Triton X-100 and 0.05% Saponin. Free aldehydes were quenched with 50 mM Glycine/Lysine in PBS. Blocking was carried out with 1% (w/v) bovine serum albumin in PBS containing 10% goat or donkey serum (EMD Millipore) for 30 min at room temperature. Cells were incubated overnight with

the following monoclonal primary antibodies: NTR3/sortilin C-20, and TREM-2 (Santa Cruz Biotech.) at 1:500 diluted, CD11b/Integrin αM, Iba1 and NTR3/sortilin (Abcam, Cambridge, MA). For immunofluorescence detection, the following secondary antibodies were used: anti-rabbit IgG-FITC (Santa Cruz Biotech.) or anti-rabbit Alexa 488 and/or anti-goat Alexa 594 (Life Technologies) at 1:1000 dilution for 2 h. Nuclei were stained with DAPI or Hoechst (50 ng/mL) and slides were then mounted using ProLong® Diamond Antifade Mountant (Life Technologies). Imaging was carried out using Nikon A1R inverted confocal microscope, with an automated stage using a conventional point scanner (1020 x 1020 pixel field view), and equipped with spectral detector for confocal scanning laser imaging, and transmitted-laser DIC detector (3 solid-state lasers and a dual-line argon gas laser, producing excitation lines at 403, 457/476, 488/514, 560 and 640 nm), and NIS-Element Software for image analysis (Nikon Instruments Inc., Melville, NY).

Protein levels of soluble NTR3/sortilin in culture medium from human microglia after stimulation by NT (10 nM) for 24 or 48 h were measured using the SORT1 ELISA (LifeSpan BioSciences, Inc, Seattle, WA). Initially, human microglia were seeded in (2×10^6 cells/ type I collagen coated T25-flask) for 24 h, serum-starved for 12 h, prior to stimulation with NT (10 nM). After 24 or 48 h, supernatant fluids were collected (10 mL) and concentrated (100 µL) using 10-K cellulose membrane centrifugal filter units (Merck Millipore, Carrigtwohill CO, Ireland). For all experiments the control cells were treated with equal volume of culture medium and the minimum detectable level for soluble NTR3/sortilin by ELISA was 0.157 ng/mL.

Pro-inflammatory Mediator Gene Expression

Microglia (1×10^5 cells/well) were seeded in 6-well, type I collagen or poly-L-lysine-coated plates (Becton Dickinson) for 24 h before stimulation with NT (1-1000 nM) or LPS (10-1000 ng/mL) (Sigma-Aldrich) was carried out for 12 h or 24 h. Total RNA was isolated as described above, and qRT-PCR was performed using Taqman gene expression assays (Applied Biosystems) to assess the expression of IL-1β, CXCL8, CCL2 and CCL5 in microglia after NT stimulation for 6 h, 12 h, or 24 h. For select experiments, microglia were pretreated with PI3K/mTOR inhibitors prior to stimulation with NT or LPS for 12 h in serum-free media before harvesting cell lysates. For all qRT-PCR studies, the suggested best coverage Taqman® probes were selected and performed. The gene levels of IL-1β, TNF, CXCL8, CCL2 and CCL5 were measured and expression was normalized to GAPDH endogenous control.

Pro-inflammatory Mediator Release

The detection of the various cytokines/chemokines within human microglia cell-conditioned culture medium/ supernatant fluid was carried out using the Human Cytokine Array Panel A (R&D Systems, Minneapolis, MN). Thereafter, specific mediator release in human microglia-conditioned culture medium was quantified by using commercial available ELISA kits (R&D Systems) as per manufacturer's instructions. Microglia (0.5×10^5 cells/well) were seeded in 12- or 24-well, type I collagen or poly-L-lysine-coated plates (Becton Dickinson) for 24 h before stimulation with NT (1-1000 nM) or LPS (10-1000 ng/mL) (Sigma-Aldrich) was carried out. For select experiments, microglia were pretreated with PI3K/mTOR for 30 min prior to stimulation with NT or LPS for 24 h in serum-free media. After 12 or 24 h, supernatant fluids were collected and IL-1, IL-6,

TNF, CXCL8, CCL2 and CCL5 release was measured. For all experiments, the control cells were treated with equal volume of culture medium and the minimum detectable level for all mediators by ELISA was 5 pg/mL.

Assessing mTOR Activation

The detection of the various signaling proteins within human microglia was carried out using the Human Phospho-Kinase Array (R&D Systems, Minneapolis, MN). The activation of mTORC1 was assessed by phosphorylation (p) of pmTOR Ser2448 and the downstream mTORC1 substrates, pp70S6K and p4EBP1, while mTORC2 activation was determined by pAKT Ser473 levels, using Western blot analysis and phospho-ELISA kits (R&D Systems). Microglia (1×10^6 cells) were seeded in 100 mm type I collagen coated dishes (Becton Dickinson) for 24 h, serum-starved overnight (or pretreated with inhibitors), then stimulated with NT (10-100 nM) for 0-60 min, before cell lysates were harvested in RIPA buffer (Sigma Aldrich), containing Halt Protease and Phosphatase Inhibitor Cocktails (Thermo Fisher Sci., Rockford, IL). The total protein concentration was determined by the bicinchoninic acid assay (Thermo Fisher Sci.) using bovine serum albumin protein (BSA) as standards. The total cellular proteins (20 or 40 µg) were separated using 4-20 % Mini-PROTEAN TGX™ precast gels (BioRad) under SDS denaturing conditions and electro-transferred onto PVDF membranes (EMD Millipore). Blocking was carried out with 5% (w/v) BSA in Tris-buffered saline (TBS) containing 0.1 % Tween-20. The membranes were probed with the following primary antibodies: mTOR (7C10), pmTOR Ser2448, mTORC1 substrates p70S6K, pp70SK Thr389, pp70SK Ser371, 4EBP1 and p4EBP1 Thr37/46, and β-actin as the loading control (Cell Signaling Technology, Danvers, MA). All proteins were visualized with

horseradish peroxidase-conjugated secondary antibodies and then by SuperSignal West Pico enhanced chemiluminescence (Thermo Fisher Sci). In parallel experiments, to quantify the levels of pAKT Ser473, pmTOR Ser2448 and pp70S6K Thr389 in microglial lysates, specific phospho-ELISA kits were used (R&D Systems).

Microglia Proliferation

Microglia (1×10^4 cells/well for 24 h or 5×10^3 cells/well for 48 h) were seeded in type-I collagen coated, 96 or 48-well flat bottom plates (Becton Dickinson) for 24 h prior to pretreatment with PI3K/mTOR inhibitors for 30 min and/or stimulation with NT (10-100 nM). All experiments were conducted in phenol-free PriGrow III media (ABM Inc.). Proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) based in vitro toxicology assay kit (Sigma Aldrich), and according to manufacturer's instructions, MTT stock solution (5 mg/ml) was added to each culture being assayed to equal one-tenth the original culture volume and incubated for 3 to 4 h, after which the converted dye was solubilized with acidic isopropanol (0.04-0.1 N HCl in absolute isopropanol). Absorbance of converted dye was measured at a wavelength of 570 nm with background subtraction at 690 nm, and % cell proliferation was calculated as percent total with control cells as 100 %.

Human Subjects

Fasting blood was obtained from Caucasian male children (n=36, 4-13 years of age) on the entire ASD, who donated blood as part of their diagnostic workup at the Orthobiotiki Polyclinic, Athens, Greece. Children were diagnosed with ASD based on clinical assessment and corroborated by meeting the cutoff scores on both the DSM-5 symptom list and the autism diagnostic observation schedule (ADOS) algorithm. They

were medication-free prior to blood draw for at least 2 weeks for all psychotropic medications and 4 weeks for fluoxetine or depot neuroleptics. The exclusion criteria were: (1) Any genetic condition linked to ASD (for example, Rett syndrome, Fragile X syndrome, tuberous sclerosis or focal epilepsy); (2) Any genetic syndrome involving the central nervous system, even if the link with ASD was uncertain; (3) Any neurologic disorder involving pathology above the brain stem, other than uncomplicated non-focal epilepsy; (4) Contemporaneous evidence, or unequivocal retrospective evidence, of probable neonatal brain damage; (5) Clinically significant visual or auditory impairment, even after correction; (6) Any severe nutritional or psychological deprivation; (7) Cutaneous or systemic mastocytosis; (8) History of allergies and upper airway diseases; (10) History of inflammatory diseases (for example, juvenile rheumatoid arthritis, inflammatory bowel disease). Oral consent was obtained from all parents. Serum was also collected from normally developing, healthy male children (n=20, 4-13 years of age) unrelated to subjects with ASD, who were seen for routine health visits at the Pediatric Department of the Social Security Administration (IKA) polyclinic. Serum samples were labeled only with a code number, the age and sex of the subjects. All ASDs and control blood samples were prepared immediately and serum was stored in -80 °C. Samples were then transported on dry ice to Boston for analysis. The detection of soluble NTR3/sortilin in serum was done using the human SORT1 ELISA (LifeSpan BioSciences, Inc. Seattle, WA).

Statistical Analysis

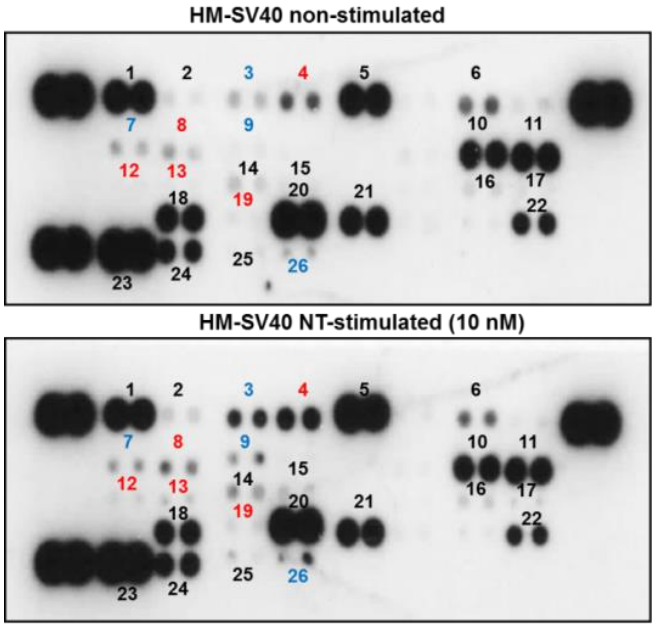
All conditions were performed in triplicate and all experiments were repeated at least three times (n=3). Results from cultured cells are presented as mean ± SD. Comparisons

were made between (1) control and stimulated cells and (2) stimulated cells with and without siRNA pre-treatment using the unpaired 2-tailed, Student's *t*-test, with significance of comparisons denoted by the horizontal lines and by $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).Comparisons were also made between (1) for all conditions with stimulated cells and with inhibitors using one-way ANOVA, followed by post-hoc analysis by Dunnett's Multiple Comparison Test; significance is denoted by horizontal lines and indicated by $p<0.001$ or $p<0.0001$ and (2) all the inhibitors/flavonoids among themselves using one-way ANOVA, followed by post-hoc analysis by Tukey's Multiple Comparison Test; those conditions for which there is significance denoted by the horizontal brackets and by the corresponding $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).Analysis of human serum samples are presented as a scattergram with symbols representing individual data points and the horizontal lines representing the mean for each group. Normality of distribution was checked with the Shapiro–Wilk's test. Comparison between the healthy control and the ASD groups was performed using Mann–Whitney U-tests and Wilcoxon matched pair test. Correlations between serum sortilin and NT levels were examined using the Spearman rank correlation test. Significance of comparisons is denoted by $p<0.0001$.The analysis was performed by using the GraphPad Prism version 5.0 software (GraphPad Software, San Diego, CA, USA). Representative images for western blots were scanned and analyzed using Image J (NIH, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2015) and confocal images by Fiji Image J.

3.3. Results

NT induces expression of pro-inflammatory cytokines and chemokines in human microglia

To evaluate whether NT can switch human microglia to the pro-inflammatory M1 phenotype, we first used a human cytokine and chemokine array blot to identify any cytokines and chemokines differentially expressed in control and NT-stimulated human microglia-SV40. NT increases expression of numerous pro-inflammatory cytokines and chemokines (Fig. 2.1), which were then measured by specific ELISAs.



1	C5/C5a
2	CD40 ligand
3	G-CSF
4	GM-CSF
5	GROα
6	sICAM-1
7	IL-1α
8	IL-1β
9	IL-1ra
10	IL-6
11	CXCL8
12	IL-10
13	IL12p70
14	1L-17
15	IL-17E
16	IL-23
17	IL-27
18	IP-10
19	CCL2
20	MIF
21	MIP-1β
22	SerpinE1
23	CCL5
24	SDF-1
25	TNFα
26	sTREM1

Figure 2.1. Identification of pro-inflammatory mediators differentially expressed in control and NT-stimulated human microglia-SV40. Immortalized HM-SV40 (10 x10^6 cells) were stimulated with NT (10 nM) for 24 h and the cultured medium/supernatants were subjected to the human cytokine and chemokine array blot to probe for the release of pro-inflammatory mediators. Increased levels of several pro-inflammatory cytokines and chemokines (blue), including IL-1beta, CXCL8, CCL2 and CCL5 (red) were denoted in NT-stimulated microglia, when compared to those from control cells. All conditions were performed in a single blot as shown.

Stimulation with NT at physiological doses (10 or 100 nM) for 24 h, increases secretion of the pro-inflammatory cytokine IL-1beta and the chemokines, CXCL8, CCL2 and CCL5 ($p<0.001$), as compared to controls, from both primary human microglia and microglia-SV40 (Fig. 2.2A-D).

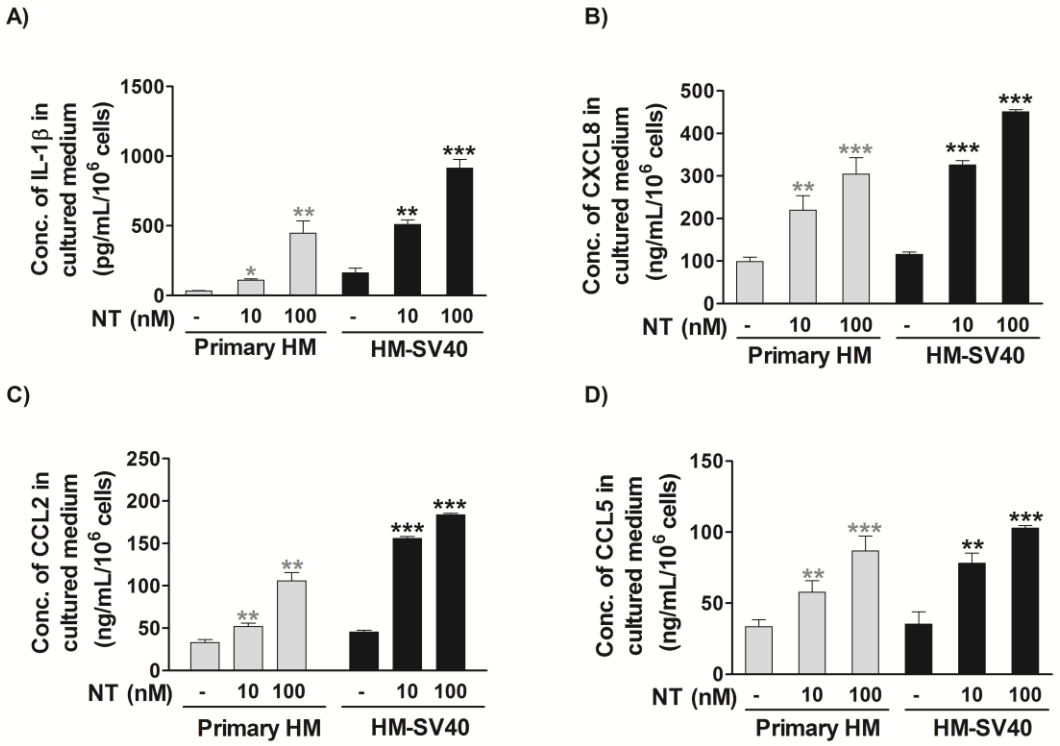


Figure 2.2. Pro-inflammatory cytokine and chemokine release from human microglia stimulated by NT. Primary human microglia (HM) (5×10^4 cells) and immortalized HM-SV40 (5×10^4 cells) were stimulated with NT (10 or 100 nM) for 24 h to measure release of (A) IL-1beta, (B) CXCL8, (C) CCL2 and (D) CCL5 by specific enzyme-linked immunosorbent assays (ELISA). All conditions were performed in triplicates for each data set and repeated three times (n=3). Significance of comparisons is denoted by $p<0.05$(*) or $p<0.001$ (**) or $p<0.0001$ (***).

LPS (10 or 100 ng/mL) used as a positive control also increases ($p<0.0001$) the secretion of all mediators, including IL-6 and TNF, from both human microglia cell types, after 24 h stimulation (Fig. 2.3).

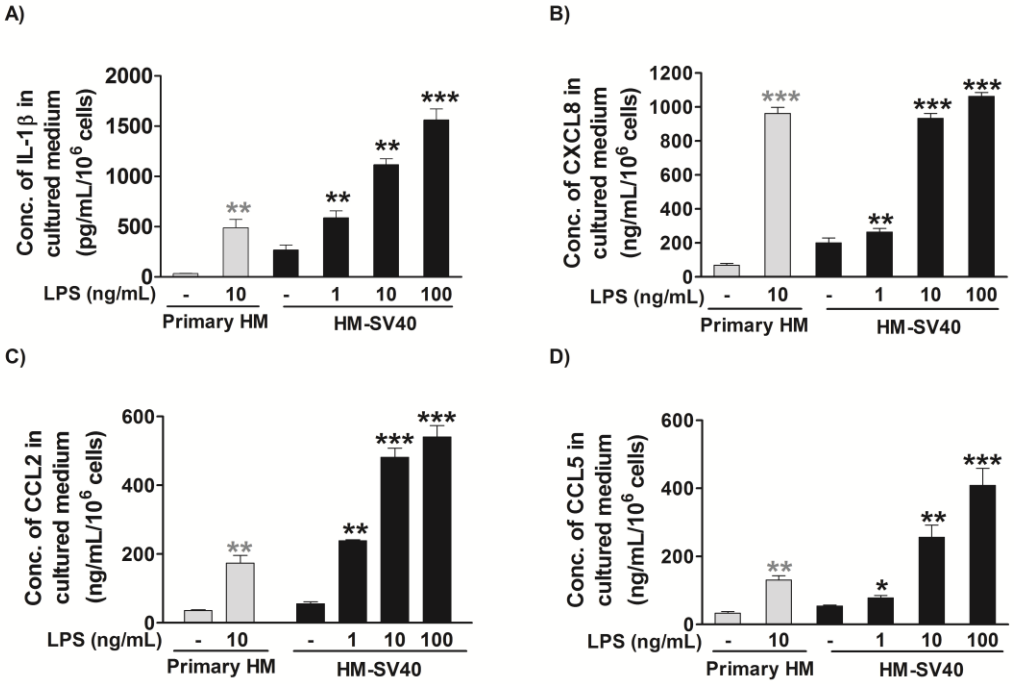


Figure 2.3. Pro-inflammatory cytokine and chemokine release from human microglia stimulated by LPS. Primary human microglia (HM) (5 x10^4 cells) and immortalized HM-SV40 (5 x10^4 cells) were stimulated with LPS (1-100 ng/mL) for 24 h to measure release of (A) IL-1beta, (B) CXCL8, (C) CCL2, (D) CCL5 by specific enzyme-linked immunosorbent assays (ELISA). All conditions were performed in triplicates for each data set and were repeated three times (n=3). Significance of comparisons is denoted by $p<0.001$ (**) or $p<0.0001$ (***).

Stimulation with NT (10 or 100 nM) also significantly increases ($p<0.0001$) the gene expression of IL-1beta, CXCL8, CCL2 and CCL5 after 12 h in both primary human microglia and microglia-SV40 (Fig. 2.4A-D). LPS (10 or 100 ng/mL, $p<0.0001$) also increases the synthesis of these mediators from human microglia, after 12 h stimulation.

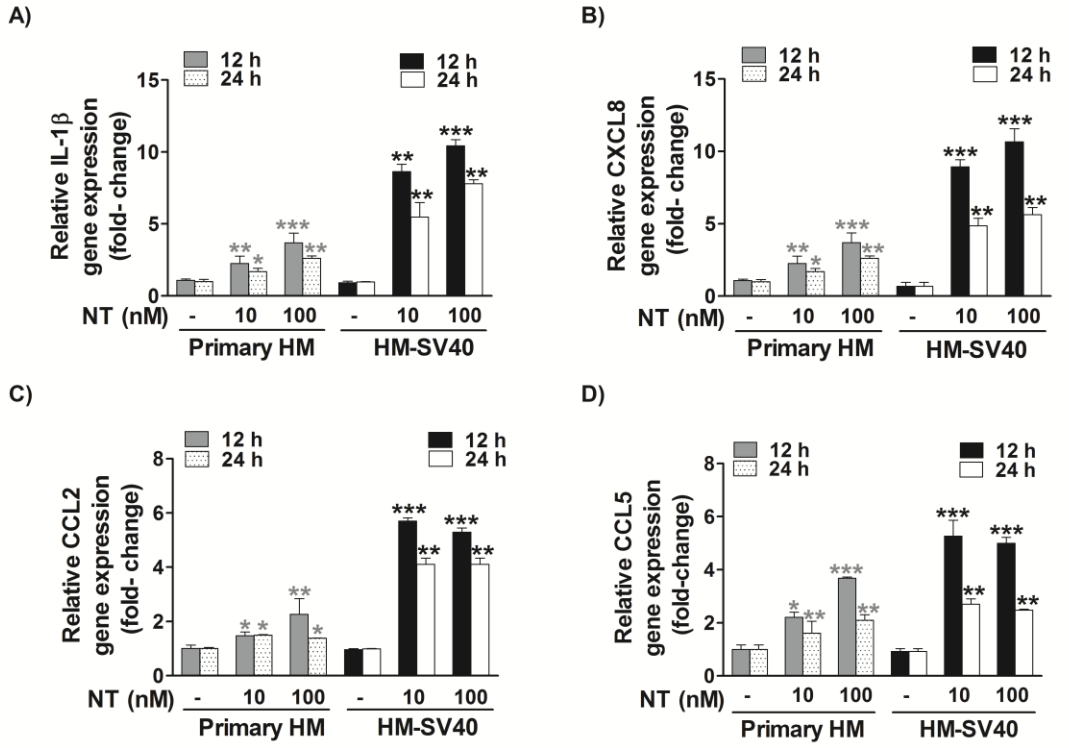


Figure 2.4. Pro-inflammatory mediator gene expression in human microglia stimulated by NT. Primary HM (2.5 x10^5 cells) and immortalized HM-SV40 (2.5 x10^5 cells) were stimulated with NT (10or 100 nM) for 12 or 24 h to determine changes in gene expression levels of (A) IL-1beta, (B) CXCL8, (C) CCL2 and (D) CCL5 by quantitative real-time PCR (qRT-PCR). All conditions were performed in triplicates for each data set and were repeated three times (n=3). Results were normalized against the endogenous gene, GAPDH, and are expressed relative to the mean of the gene of interest. Significance of comparisons is denoted by $p<0.05$(*) or $p<0.001$ (**) or $p<0.0001$ (***).

Human microglia express only NTR3/sortilin, which increases in gene and surface protein localization in response to NT

We next investigated the expression of the three types of NT receptors, NTR1, NTR2 and NTR3/sortilin in both primary human microglia and the human microglia-SV40 cell line.

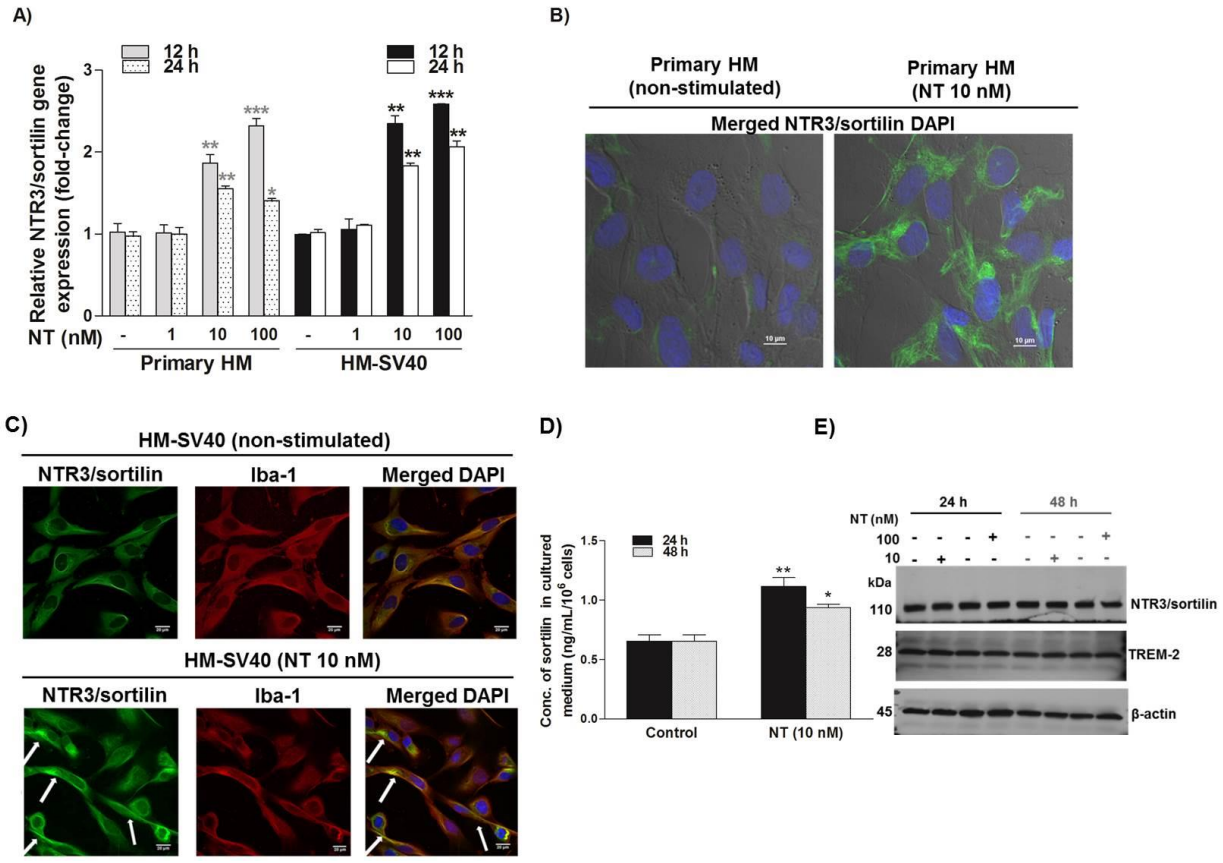


Figure 2.5. NTR3/sortilin gene expression and cellular localization in human microglia, and its release in response to NT. Primary HM (2.5 x10^5 cells) and immortalized HM-SV40 were stimulated with NT for 12 or 24 h to measure (A) gene expression levels of NTR3/sortilin in control and NT-stimulated (10 or 100 nM) microglia by qRT-PCR. All conditions were performed in triplicates for each data set and were repeated three times (n=3). Results were normalized against the endogenous GAPDH and expressed relative to the mean of the control for the gene of interest, with significance of comparisons denoted by $p<0.05$(*) or $p<0.001$ (**) or $p<0.0001$ (***). Microglia-SV40 (5×10^3 cells/four-well chamber coated slide) were stimulated with NT (10 nM) for 24 h, then fixed and permeabilized to stain for nuclei using DAPI (blue), with specific antibodies for Alexa 488-NTR3/sortilin (green) or Alexa 594-Iba-1, a microglial-marker protein (red), while rabbit IgG was used for the negative control. The cell surface and cytosolic distribution of NTR3/sortilin protein is shown in control and NT-stimulated (B) primary HM and (C) immortalized HM-SV40, where colocalization with Iba1 is also apparent (white arrows). Qualitative analysis was done using images from triplicates and representative images are shown. (D) HM-SV40 (1 x10^6 cells) were stimulated with NT (10 nM) for 24 or 48 h to measure release of soluble NTR3/sortilin in culture media by ELISA. All conditions were performed in triplicates for each data set and repeated three times (n=3). Significance of comparisons is denoted by $p<0.05$(*) or $p<0.001$ (**). E) HM-SV40 (1 x10^6 cells) were stimulated with NT (10 or 100 nM) for 24

or 48 h to measure total cellular NTR3/sortilin levels by Western blot analysis, where TREM-2 served as a microglial-marker protein and β-actin as the loading control. All conditions were performed in triplicates for each data set and repeated three times (n=3) and representative image is shown.

The high affinity NTR1 and low affinity NTR2 are undetectable; however, NTR3/sortilin gene expression is detectable, and significantly increases ($p<0.001$) after 12 or 24 h of NT treatment (10 or 100 nM) (Fig. 2.5A). Protein levels of NTR1 and NTR2 are also undetectable by Western blot analysis.

To determine changes in the cellular localization of NTR3/sortilin after NT stimulation, DIC/confocal immunofluorescence microscopy was carried out. Immunodetectable NTR3/sortilin in control primary human microglia and microglia-SV40 reveals a cytosolic distribution. Stimulation with NT (10 nM) for 24 h increases the surface associated NTR3/sortilin in primary human microglia (Fig. 2.5B) and in the microglia-SV40, which appears to be co-localized with the filamentous actin binding protein, ionized calcium binding adaptor molecule-1 (Iba-1) (Fig. 2.5C).

We hypothesized that surface NTR3/sortilin may also be secreted extracellularly. Stimulation of human microglia-SV40 with NT (10 nM) for 24 h ($p<0.0001$) or 48 h ($p<0.05$) increases levels of soluble NTR3/sortilin (Fig. 2.5D). There is no apparent significant difference in the total cellular NTR3/sortilin levels in human microglia stimulated with NT (10 or 100 nM) after 24 or 48 h, compared to control cells (Fig. 2.5E).

NT stimulates pro-inflammatory cytokine and chemokine release from human microglia via NTR3/sortilin

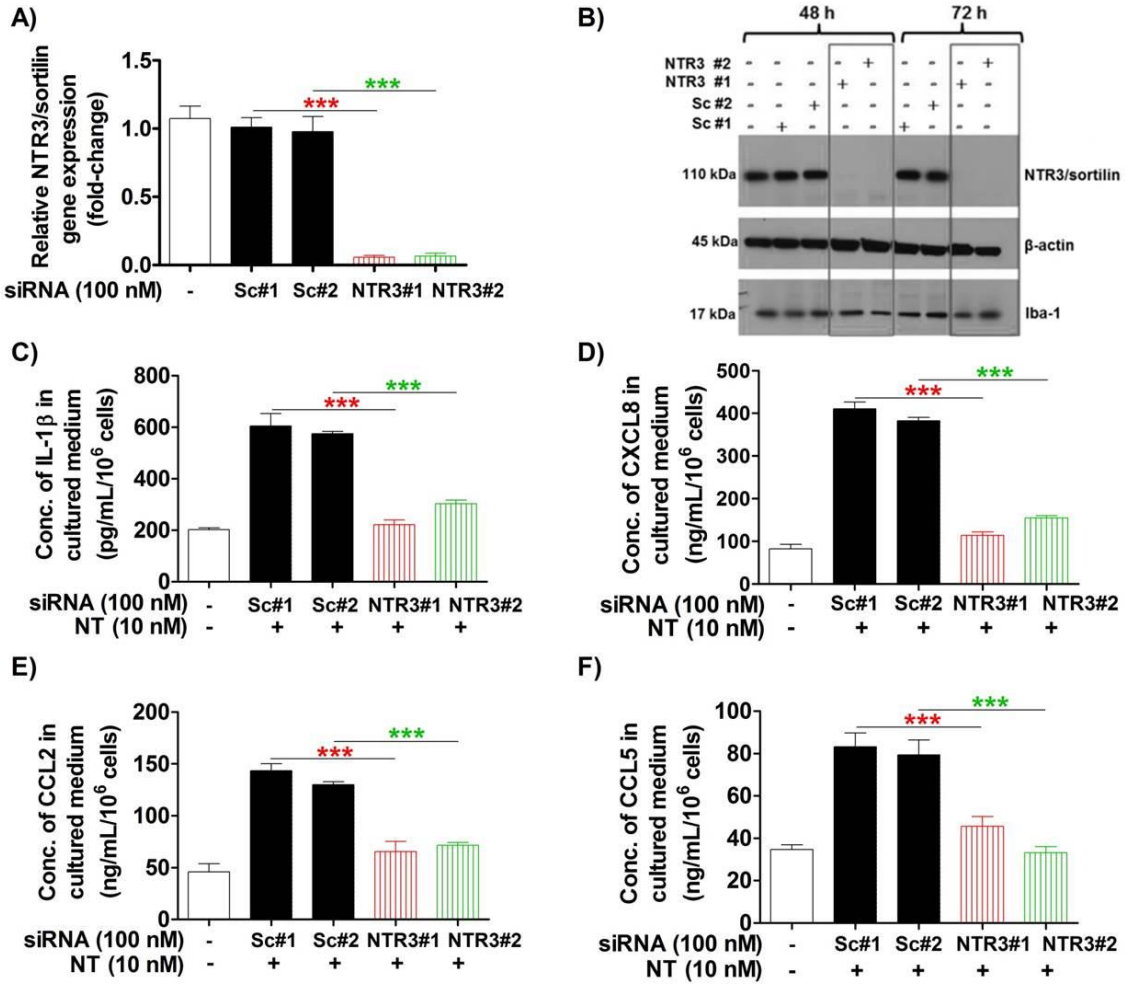


Figure 2.6. NT induces pro-inflammatory mediator release from human microglia via NTR3/sortilin. Immortalized HM-SV40 ($1x10^6$ cells) were transfected with two different predesigned and validated siRNAs targeting human NTR3/sortilin (NTR3 #1 and #2) or scramble (Sc) controls (Sc #1 and Sc #2) for 48 h and 72 h prior to evaluation of NTR3/sortilin (A) gene levels by qRT-PCR and (B) protein levels by Western blot analysis. Control and siRNA transfected microglia-SV40 ($5 x10^4$ cells) were stimulated with NT (10 nM) for 24 h to measure release of (C) IL-1beta, (D) CXCL8, (E) CCL2 and (F) CCL5 by ELISA. All conditions were performed in triplicates for each data set and were repeated three times (n=3). Significance of comparisons is denoted by $p<0.0001$ (***).

Even though human microglia do not express NTR1 and NTR2, we pre-treated microglia-SV40 with the NTR1 selective non-peptide antagonist SR48692 or the dual NTR1/NTR2 antagonist SR142948A (10-1000 nM for 1 h), prior to stimulation with NT (10 or 100 nM). Pre-treatment with these receptor antagonists did not affect IL-1beta, CXCL8, CCL2 and CCL5 mediator release from human microglia-SV40. In order to determine whether NTR3/sortilin mediates the NT-stimulated pro-inflammatory cytokine and chemokine release, human microglia were subjected to siRNA downregulation of NTR3/sortilin levels. Human microglia-SV40 were transfected with two different scramble and targeted NTR3/sortilin siRNAs. Gene expression analysis by qRT-PCR revealed >95% knockdown of NTR3/sortilin in siRNA-transfected cells after 48 h, compared to control siRNA transfected or unstimulated microglia (Fig. 2.6A).

Protein levels of NTR3/sortilin in microglia-SV40 transfected with NTR3 siRNA are abolished, while the control cells treated with scramble siRNA retain normal expression levels after 48 or 72 h (Fig. 2.6B). Stimulation by NT of microglia in which NTR3/sortilin levels were down-regulated, significantly decreases ($p<0.001$) IL-1beta, CXCL8, CCL2 and CCL5 release, as compared to control cells (Fig. 2.6C-F).

In order to ensure that the effect of downregulated NTR3/sortilin was not due to any involvement in intracellular mediator transport or release, we investigated the level of pro-inflammatory mediator gene expression in NT-stimulated scramble and targeted NTR3/sortilin siRNA treated microglia SV40. Gene expression of IL-1beta, CXCL8, CCL2 and CCL5 significantly decreases ($p<0.001$) after NT stimulation in microglia with downregulated NTR/sortilin levels, compared to scramble siRNA-treated or unstimulated microglia (Fig. 2.7).

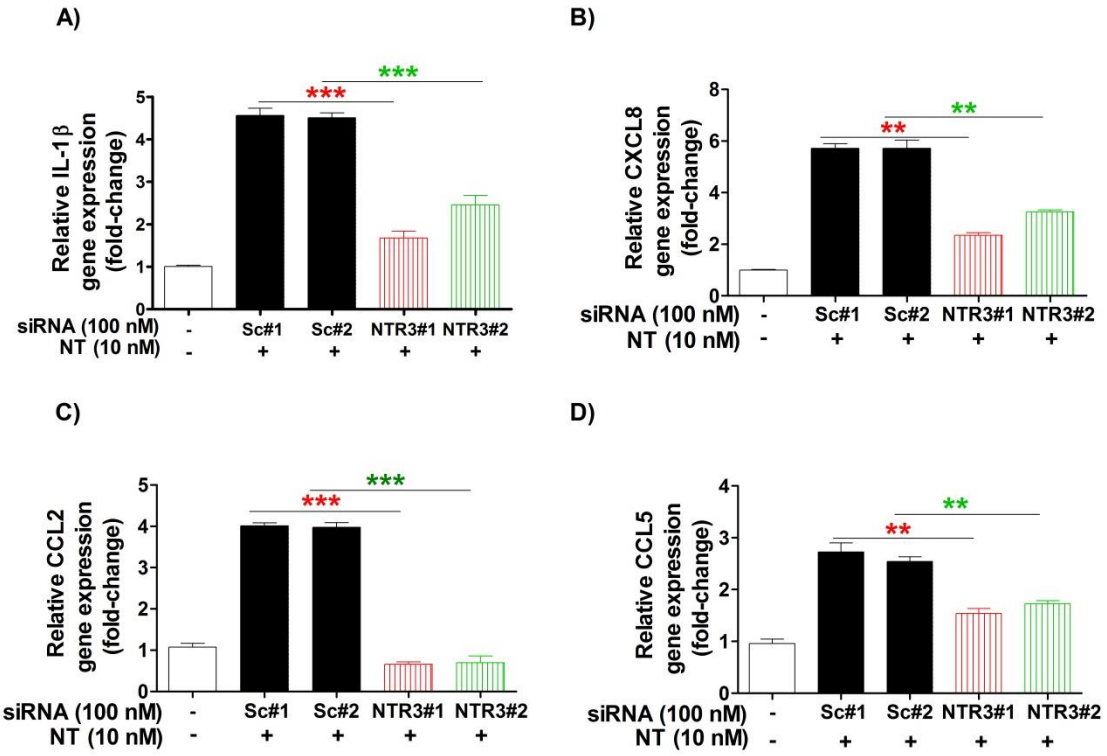
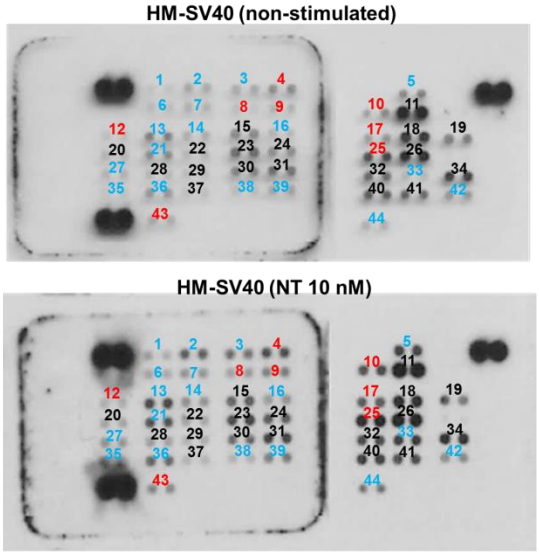


Figure 2.7. Pro-inflammatory mediator gene expression in NT stimulated human microglia pretreated with control scramble and NTR3/sortilin siRNA. Control and siRNA transfected microglia-SV40 (2 x10^6 cells) were stimulated with NT (10 nM) for 24 h and culture medium concentrated to measure release of (A) IL-1beta, (B) CXCL8, (C) CCL2 and (D) CCL5 by qRT-PCR. All conditions were performed in triplicates for each data set and were repeated three times (n=3). Results were normalized against the endogenous GAPDH and expressed relative to the mean of the control for the gene of interest, with significance of comparisons denoted by $p<0.05$(*) or $p<0.001$ (**) or $p<0.0001$ (***).

NT activates PI3K/mTOR signaling in human microglia that is blocked by luteolin and methoxyluteolin

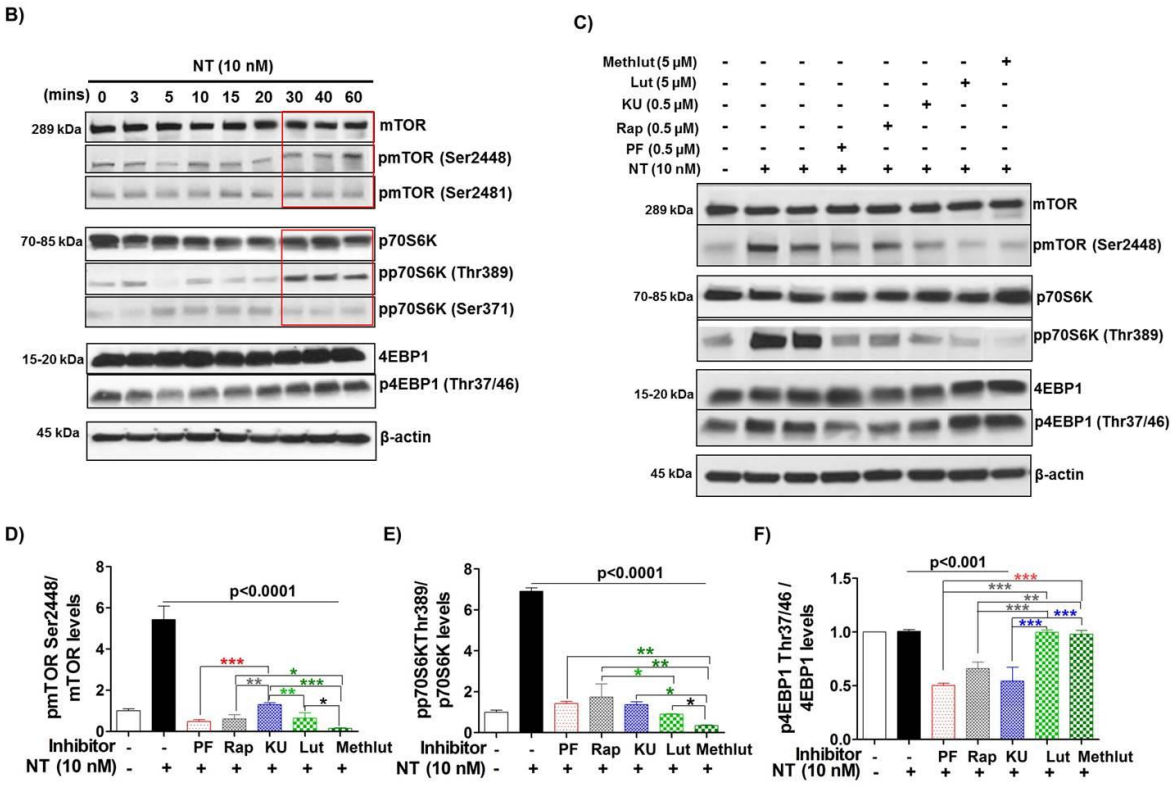
To investigate the signaling pathway involved in the stimulation of human microglia-SV40 in response to NT, a phospho-array blot to detect the phosphorylated signaling proteins was used.



1	p38α (T180/Y182)	23	STAT2 (Y689)
2	ERK1/2 (T202/Y204, T185/Y187)	24	STAT5a (Y694)
3	JNK1/2/3 (T183/Y185,T221/Y223)	25	p70S6Kinase (T421/S424)
4	GSK-3α/β (S21/S9)	26	RSK 1/2/3 (S380/S386/S377)
5	p53 (S392)	27	Fyn (Y420)
6	EGF R (Y1086)	28	Yes (Y426)
7	MSK1/2 (S376/S60)	29	Fgr (Y412)
8	AMPKα1 (T183)	30	STAT6 (Y641)
9	AKT 1/2/3 (S473)	31	STAT5b (Y699)
10	AKT 1/2/3 (T308)	32	STAT3 (Y705)
11	P53 (S46)	33	p27 (T198)
12	TOR (S2448)	34	PLC-γ1 (Y783)
13	CREB (S133)	35	HcK (Y411)
14	HSP27 (S78/S82)	36	Chk-2 (T68)
15	AMPKα2 (T172)	37	FAK (Y397)
16	β-Catenin	38	PDGF Rβ (Y751)
17	p70S6 kinase (T389)	39	STAT5a/b (Y694/Y699)
18	P53 (S15)	40	STAT3 (S727)
19	c-Jun (S63)	41	WNK1 (T60)
20	Src (Y419)	42	PYK2 (Y402)
21	Lyn (Y397)	43	PRAS40 (T246)
22	Lck (Y394)	44	HSP60

Figure 2.8. NT-stimulation of human microglia-SV40 involves activation of mTOR signaling, which is inhibited by luteolin and methoxyluteolin. (A) Immortalized HM-SV40 (10 x10^6 cells) were serum starved overnight, then stimulated with NT (10 nM) for 30 min, before cellular lysates were harvested to probe for the phosphorylated-levels of intracellular signaling kinases were detected , as shown using a human phospho-array blot. Increased levels of several phospho-proteins (blue), including components of the mTOR signaling pathway (red) were denoted in NT-stimulated microglia, after qualitative comparisons to control cells were made. All conditions were performed in the single blot shown (n=1).

Protein levels of phosphorylated (p) substrates that are upregulated in microglia-SV40 after NT stimulation include the downstream mTOR substrates, p70S6K and 4EBP1 proteins (Fig. 2.8A). Western blot analysis was then performed to detect the total and p-levels of mTOR, as well as p70S6K and 4EBP1 proteins after stimulation by NT (10 nM) from 0-60 min. NT increases the levels of pmTOR Ser2448 and the downstream mTORC1 substrate, pp70S6K Thr389 within 30 min (Fig. 2.8B).



(B) HM-SV40 ($1x10^6$ cells) were stimulated with NT (10 nM) for 0-60 min. The total and phosphorylated levels of mTOR and mTOR substrates p70S6K (increase after 30 min of NT-stimulation, red box) and 4EBP1 were analyzed by Western blot analysis, for which β-actin served as the loading control. (C) HM-SV40 were pre-incubated with the dual PI3K/mTOR inhibitor (PF, 0.5 μM) and the mTOR (rapamycin, Rap, 24 h or KU, 0.5 μM) inhibitors or flavonoids [luteolin (Lut) and methoxyluteolin (Methlut), 5 μM] in serum-free media overnight, then stimulated with NT (10 nM) for 30 min. The total and phosphorylated levels of mTOR and mTOR substrates were assessed by Western blot analysis. Results from densitometric analysis are presented as normalized phosphorylated to total protein levels of (D) mTOR and pmTOR Ser2448, (E) p70S6K and pp70S6K Thr389, and (F) 4EBP1 and p4EBP1 Thr37/46. All conditions were performed in triplicates for each data set and were repeated three times (n=3). Significance of comparisons were made for stimulated cells and those with inhibitors/flavonoids, as denoted by the horizontal lines ($p<0.0001$ or $p<0.001$) and also among each of the inhibitors/flavonoids treatments shown by the horizontal brackets and corresponding $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).

We next investigated whether the natural flavonoids, luteolin and methoxyluteolin, affect mTOR signaling by comparing their inhibitory effect to that of various mTOR inhibitors in microglia stimulated by NT. We used the first-generation allosteric mTOR inhibitor, rapamycin, the new small-molecule ATP-competitive kinase inhibitor of mTORC1 and mTORC2, KU-0063794 (KU) and the dual PI3K/mTOR inhibitor, PF-04691502 (PF). Human microglia-SV40 were serum-starved overnight and pre-incubated with rapamycin, KU PF, 0.5 μM or luteolin and methoxyluteolin (5 μM) for 12 h prior to NT stimulation (30 min) (Fig. 2.8C), which significantly decreases levels pmTOR Ser2448 and p70S6K Thr389, compared to those in microglia stimulated by NT (Fig. 2.8D and E). Neither NT, nor the flavonoids have any effect on the levels of total or p4EBP1 proteins (Fig. 2.8F).

Phospho-ELISAs were also performed on microglia stimulated with NT and/or pre-treated with the PI3K/mTOR inhibitors or luteolin and methoxyluteolin to quantify levels of pAKT Ser473, pmTOR Ser2448 and pp70S6K Thr389 in order to assess activation of mTOR. The levels of phosphorylated AKT or mTOR or p70S6K proteins (Fig.2.9) in microglia pre-treated with inhibitors prior to NT stimulation significantly decrease ($p<0.001$), as compared to NT stimulation. Noteworthy, methoxyluteolin (5 μM) shows greater reduction of phosphorylated levels of pmTOR Ser 2448 (Fig. 2.9B) and pp-70S6K Thr389 (Fig. 2.9C) compared to luteolin (5 μM) or any of the other inhibitors.

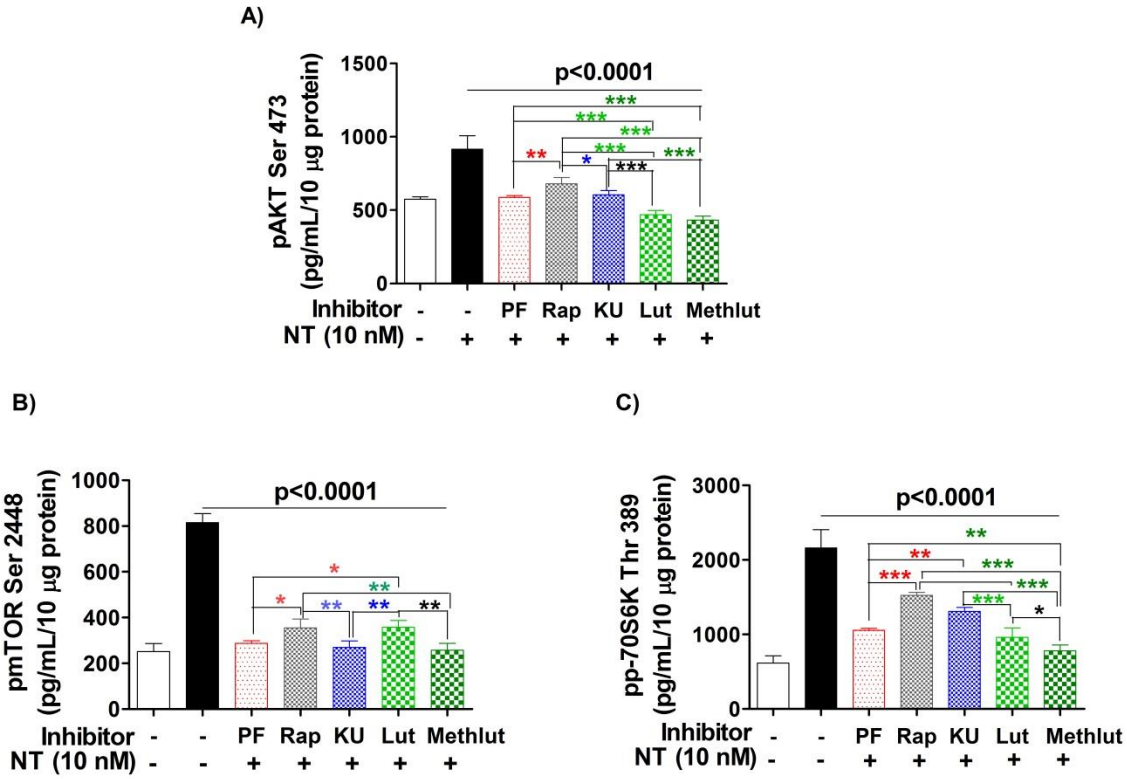


Figure 2.9. Methoxyluteolin targets inhibition of PI3K/AKT/mTOR activation in human microglia-SV40 in response to NT stimulation. HM-SV40 (1 x10^6 cells) were serum starved and pretreated with PI3K/mTOR inhibitors (0.5 µM) and the flavonoids [luteolin (Lut) and methoxyluteolin (Methlut), 5 µM] overnight, then stimulated with NT (10 nM) for 30 min to measure the protein levels of the mTORC2 substrate, (A) pAKT Ser473, (B) p mTOR Ser2448 and the downstream mTORC1 substrate (C) pp70S6K Thr389 using specific phospho-ELISA kits for equal amounts of protein. All conditions were performed in triplicates for each data set and were repeated three times (n=3). Significance of comparisons were made for stimulated cells and those with inhibitors/flavonoids, as denoted by the horizontal lines ($p<0.0001$) and also among each of the inhibitors/flavonoids treatments shown by the horizontal brackets and corresponding $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).

NT-induced pro-inflammatory cytokine and chemokine expression in human microglia dependent on mTOR activation is inhibited by luteolin and methoxyluteolin

Human microglia-SV40 were serum-starved overnight, and pretreated with PI3K and/or mTOR inhibitors (0.1-1 µM, 2 h) or with luteolin and methoxyluteolin (0.1-10 µM, 2 h), then stimulated by NT (10 nM) for 24 h to measure release of cytokines and

chemokines in serum-free media. The release of IL-1beta, CXCL8, CCL2 and CCL5 significantly decreases ($p<0.001$) in the presence of the dual PI3K/mTOR inhibitor PF, as well as the mTOR inhibitors, rapamycin and KU at the optimal inhibitory concentration of 0.5 µM, and by the flavonoids, luteolin and methoxyluteolin (5 µM) (Fig. 2.10).

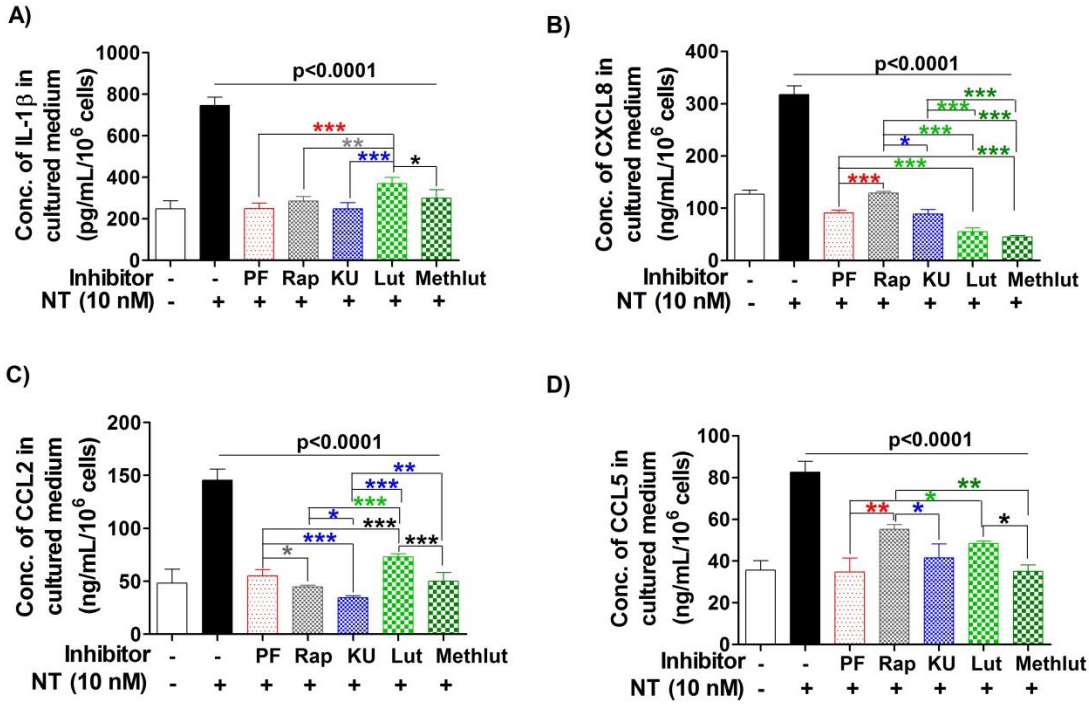


Figure 2.10. Microglia pro-inflammatory mediator release in response to NT is attenuated by the PI3K/mTOR inhibitors and the flavonoids luteolin and methoxyluteolin. HM-SV40 (5 x10^4 cells cells) were pre-treated with the dual PI3K/mTOR (PF, 0.1 µM)) and the mTOR (rapamycin, Rap and KU, 0.1 µM) inhibitors or flavonoids [luteolin (Lut) and methoxyluteolin (Methlut), 5 µM] for 2 h, then stimulated with NT (10 nM) for 24 h in serum-free medium to measure release of **(A)** IL-1beta, **(B)** CXCL8, **(C)** CCL2 and **(D)** CCL5 by ELISA. All inhibitors were dissolved in water or DMSO with final concentration < 0.1 %. All conditions were performed in triplicates for each data set and were repeated three times (n=3). Significance of comparisons were made for stimulated cells and those with inhibitors/flavonoids, as denoted by the horizontal lines ($p<0.0001$) and also among each of the inhibitors/flavonoids treatments shown by the horizontal brackets and corresponding $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).

As a positive control, the release of pro-inflammatory cytokine IL-1beta and the chemokines CXCL8, CCL2 and CCL5 mediators were measured in response to LPS (10 ng/mL), which also significantly decreases ($p<0.001$) in the presence of PI3K and/or mTOR inhibitors, as well as luteolin or methoxyluteolin (Fig. 2.11).

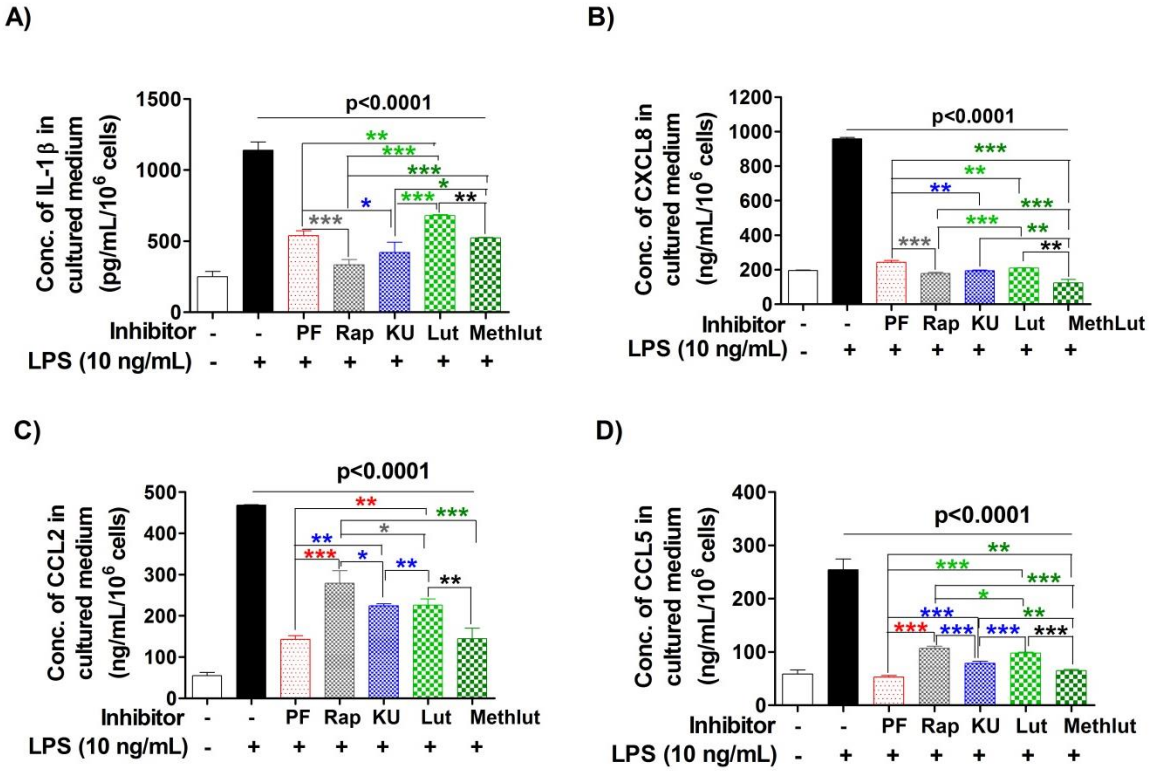


Figure 2.11. Microglia pro-inflammatory mediator release in response to LPS is attenuated by the PI3K/mTOR inhibitors and the flavonoids luteolin and methoxyluteolin. HM-SV40 (5 x10^4 cells) were pre-treated with the dual PI3K/mTOR (PF, 0.5 µM) and the mTOR inhibitors (rapamycin, Rap and KU, 0.5 µM), as well as the flavonoids [luteolin(Lut) and methoxyluteolin(Methlut), 5 µM in upper panel, or 0.1-10 µM in lower panel] for 30 min, then stimulated with NT (10 nM) for 24 h in serum-free media to measure release of **(A)** IL-1beta, **(B)** CXCL8 **(C)** CCL2 and **(D)** CCL5 by ELISA. All inhibitors were dissolved in water or DMSO with final concentration < 0.1 %. All conditions were performed in triplicates for each data set and were repeated three times (n=3). Significance of comparisons were made for stimulated cells and those with inhibitors/flavonoids, as denoted by the horizontal lines ($p<0.0001$) and also among each of the inhibitors/flavonoids treatments shown by the horizontal brackets and corresponding $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).

Methoxyluteolin is a more potent inhibitor than luteolin at equimolar flavonoid concentrations for the release of pro-inflammatory cytokine and chemokines from either NT-stimulated microglia (Fig. 2.12) or LPS-stimulated microglia (Fig. 2.13), with maximal flavonoid inhibition at 10 µM.

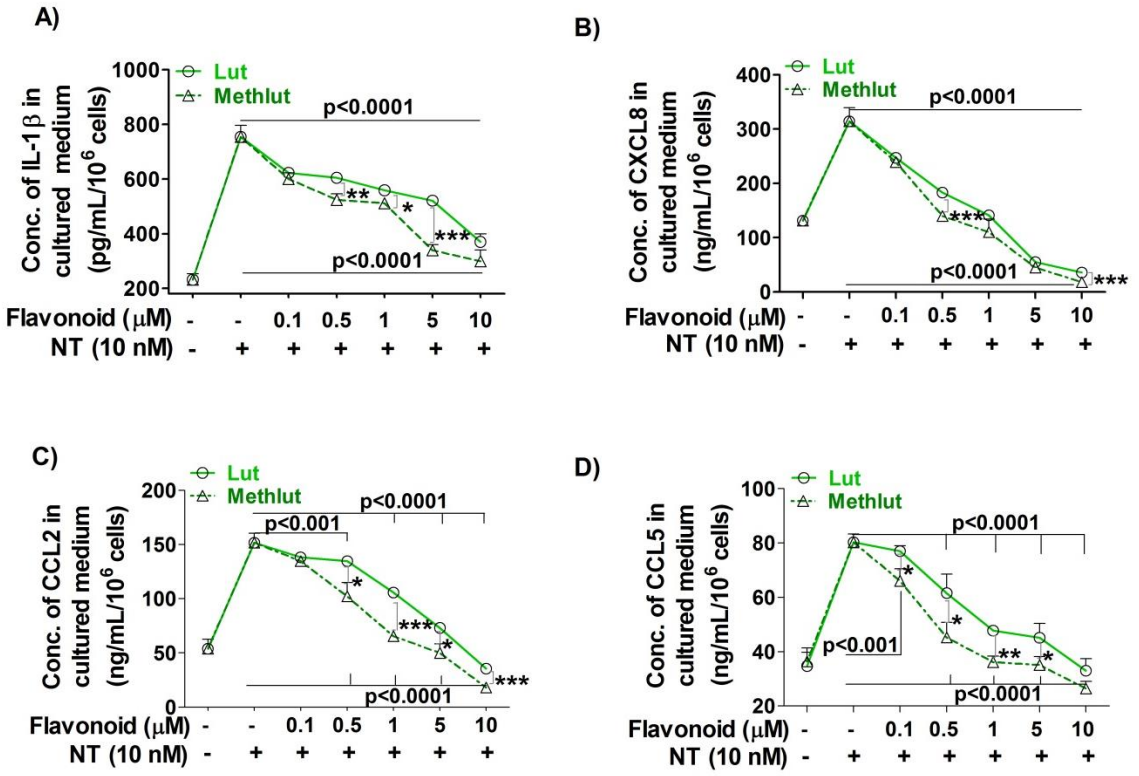


Figure 2.12. Microglia pro-inflammatory mediator release in response to NT is inhibited by flavonoids methoxyluteolin better than luteolin. HM-SV40 (5 x10^4 cells cells) were pre-treated with flavonoids [luteolin (Lut) and methoxyluteolin (Methlut), 0.1-10 µM] for 2 h, then stimulated with NT (10 nM) for 24 h in serum-free medium to measure release of (A) IL-1beta, (B) CXCL8, (C) CCL2 and (D) CCL5 by ELISA. All inhibitors were dissolved in water or DMSO with final concentration < 0.1 %. All conditions were performed in triplicates for each data set and were repeated three times (n=3). Significance of comparisons were made for stimulated cells and those with flavonoids Methlut (top) and Lut (bottom) as denoted by the horizontal lines ($p<0.0001$) and also among each of the flavonoid treatments shown by the vertical brackets and corresponding $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).

We next investigated whether mTOR signaling is involved in the production of pro-inflammatory cytokines and chemokines in human microglia after NT stimulation and whether luteolin and methoxyluteolin can inhibit these responses. The gene expression of IL-1beta, CXCL8, CCL2 and CCL5 in microglia pretreated with PI3K and/or mTOR inhibitors (0.1-1 µM) and luteolin or methoxyluteolin (0.1-10 µM) for 2 h, prior to stimulation by NT (10 nM) for 12 h were measured.

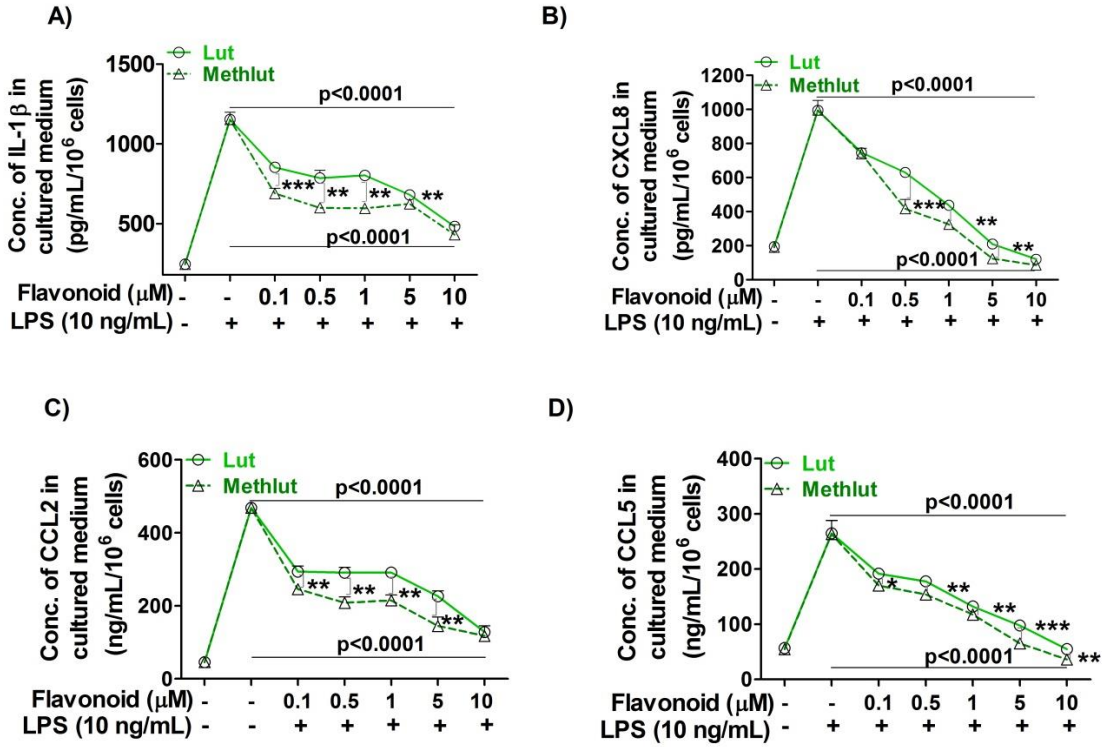


Figure 2.13. Microglia pro-inflammatory mediator release in response to LPS is inhibited by flavonoids methoxyluteolin better than luteolin. HM-SV40 (5 x10^4 cells cells) were pre-treated with flavonoids [luteolin (Lut) and methoxyluteolin (Methlut), 0.1-5 µM] for 2 h, then stimulated with LPS (10 ng/mL) for 24 h in serum-free medium to measure release of (A) IL-1beta, (B) CXCL8, (C) CCL2 and (D) CCL5 by ELISA. All inhibitors were dissolved in water or DMSO with final concentration < 0.1 %. All conditions were performed in triplicates for each data set and were repeated three times (n=3). Significance of comparisons were made for stimulated cells and those with flavonoids Methlut (top) and Lut (bottom) as denoted by the horizontal lines ($p<0.0001$) and also among each of the flavonoid treatments shown by the vertical brackets and corresponding $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).

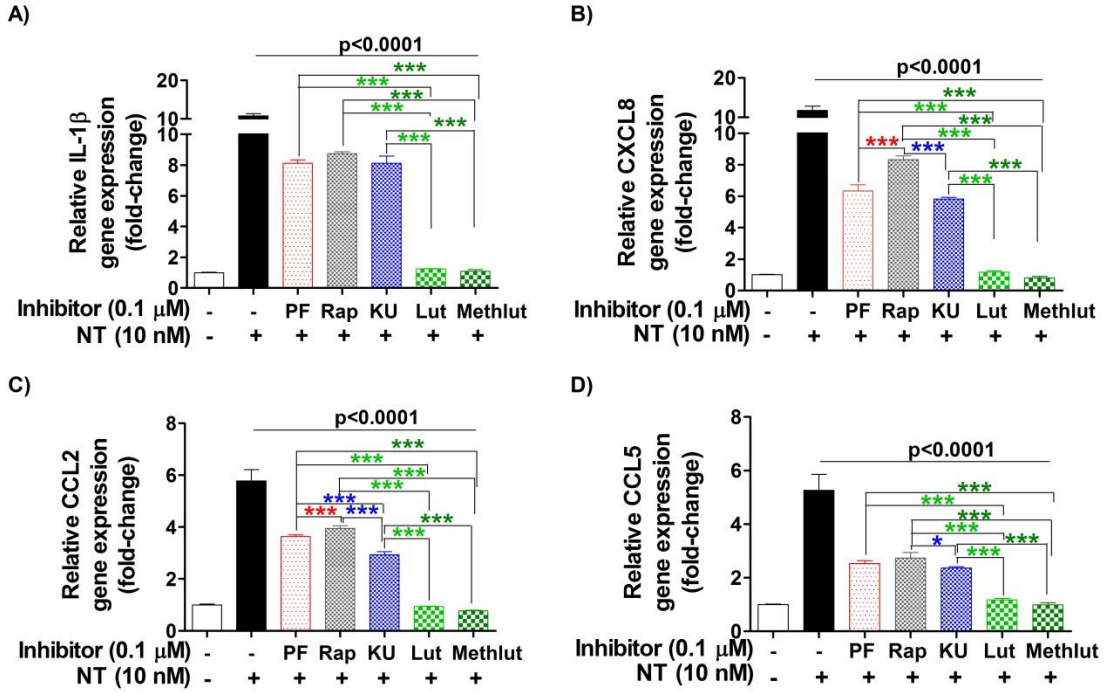


Figure 2.14. Microglia pro-inflammatory mediator gene expression in response to NT is attenuated by the PI3K/mTOR inhibitors and the flavonoids luteolin and methoxyluteolin. HM-SV40 (5 x10^4 cells cells) were pre-treated with the dual PI3K/mTOR (PF, 0.1 μM) and the mTOR (rapamycin, Rap and KU, 0.1 μM) inhibitors or the flavonoids [luteolin (Lut) and methoxyluteolin (Methlut), 0.1 μM] for 2 h, then stimulated with NT (10 nM) for 12 h in serum-free media to determine changes in gene levels of (A) IL-1beta, (B) CXCL8, (C) CCL2 and (D) CCL5 by qRT-PCR. All inhibitors were dissolved in water or DMSO with final concentration < 0.1 %. Results were normalized against the endogenous gene, GAPDH and are expressed relative to the mean of the gene of interest. All conditions were performed in triplicates for each data set and were repeated three times (n=3). Significance of comparisons were made for stimulated cells and those with inhibitors/flavonoids, as denoted by the horizontal lines ($p<0.0001$) and also among each of the inhibitors/flavonoids treatments shown by the horizontal brackets and corresponding $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).

Pre-treatment with PI3K and/or mTOR inhibitors and luteolin or methoxyluteolin (0.1 μM) significantly decreases ($p<0.001$) all cytokine and chemokine gene levels, even after stimulation by NT (Fig. 12.14) or LPS (Fig. 12.15). Methoxyluteolin (0.1 μM) more

potently than luteolin inhibits the gene expression of CXCL8, CCL2 and CCL5 in human microglia-SV40 stimulated with NT.

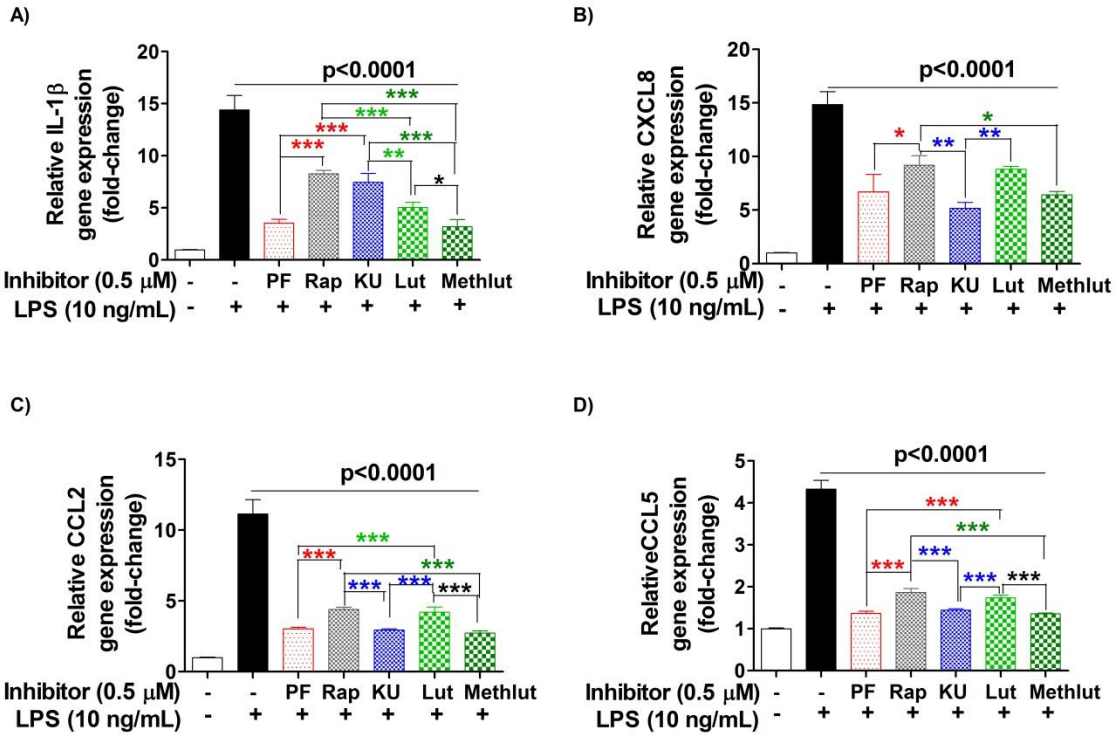


Figure 2.15. Microglia pro-inflammatory mediator gene expression in response to LPS is attenuated by the PI3K/mTOR inhibitors and the flavonoids luteolin and methoxyluteolin. HM-SV40 (2.5 x10^5 cells) were pre-treated with the dual PI3K/mTOR (PF, 0.5 μM) and the mTOR (rapamycin, Rap and KU, 0.1 μM) inhibitors and the flavonoids [luteolin (Lut) and methoxyluteolin (Methlut), 0.1 μM] for 2 h, then stimulated with LPS (10 ng/mL) for 12 h in serum-free media to determine changes in gene levels of (A) IL-1beta, (B) CXCL8, (C) CCL2 and (D) CCL5 by qRT-PCR. All inhibitors were dissolved in water or DMSO with final concentration < 0.1 %. All conditions were performed in triplicates for each data set and were repeated three times (n=3). Results were normalized against the endogenous gene, GAPDH and are expressed relative to the mean of the gene of interest. Significance of comparisons were made for stimulated cells and those with inhibitors/flavonoids, as denoted by the horizontal lines ($p<0.0001$) and also among each of the inhibitors/flavonoids treatments shown by the horizontal brackets and corresponding $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).

NT stimulated-proliferation of human microglia is dependent on mTOR signaling that is inhibited by luteolin and methoxyluteolin

Cellular proliferation of human microglia-SV40 stimulated with NT (10 or 100 nM) was assessed by the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 24 or 48 h. Proliferation of microglia increases ($p<0.001$) after 48 h stimulation by NT (10 nM), when compared to unstimulated microglia (Fig. 2.16A) and not after 24 h.

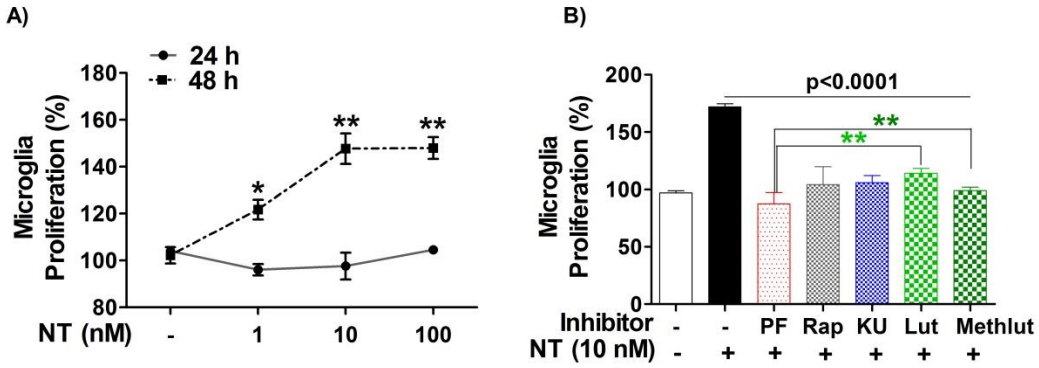


Figure 2.16. NT-stimulated proliferation of human microglia depends on mTOR signaling and is inhibited by luteolin and methoxyluteolin. (A) HM-SV40 (5×10^3 cells/well for 48 h or 10×10^3 cells/well for 24 h) were stimulated with NT (10 or 100 nM) in phenol-free medium and proliferation was measured using the MTT-based assay. Absorbance of converted dye was measured at a wavelength of 570 nm with background subtraction at 690 nm; cell proliferation was calculated with control cells set at 100 %. (B) Microglia (5×10^3 cells/well) were pretreated with the dual PI3K/mTOR (PF, 0.5 µM) and the mTOR [rapamycin (Rap) and KU, 0.5 µM] inhibitors or the flavonoids [luteolin (Lut) and methoxyluteolin (Methlut), 5 µM] for 2 h, then stimulated with NT (10 nM) for 48 h, and MTT assay was performed. All conditions were done in triplicates for each data set and were repeated three times (n=3). Results are expressed as % cell proliferation relative to the control cells, with significance of comparisons made for control and stimulated cells, as denoted by $p<0.05$(*) or $p<0.001$ (**). In (B) multiple comparisons were made for stimulated cells and those with inhibitors/flavonoids, as denoted by the horizontal lines ($p<0.0001$) and also among each of the treatments shown by the horizontal brackets and corresponding $p<0.001$ (**).

To evaluate the involvement of mTOR signaling in cellular proliferation, microglia-SV40 were pre-treated with the PI3K and/or mTOR inhibitors or luteolin and methoxyluteolin for 2 h prior to stimulation by NT (10 nM). The PI3K and/or mTOR inhibitors (0.5 μM) and luteolin or methoxyluteolin (5 μM) decrease ($p<0.001$) NT-stimulated proliferation of microglia after 48 h (Fig. 2.16B).

Increased levels of serum NTR3/sortilin is detected in the serum of children with ASD

In view of the increased NT in the serum of children with ASD and the extracellular secretion of soluble NTR3/sortilin from NT-stimulated microglia, we measured soluble sortilin in the serum of children with ASD, as compared to healthy controls. Increased levels ($p<0.0001$) of soluble NTR3/sortilin are detected in the serum of children with ASD (mean levels of normal controls: 0.9063 ng/mL ± 0.895; n=36), as compared to age- and sex-matched healthy controls (0.1395 ng/mL ± 0.094; n=20) (Fig. 2.17A). In attempt to see if there was a correlation between serum soluble NTR3/sortilin and circulating NT, levels of these proteins were measured in the same group of ASD patients and controls. There is a significant positive correlation between serum NTR3/sortilin and NT levels (Spearman's $r=0.3940$, $p=0.0283$) (Fig. 2.17B).

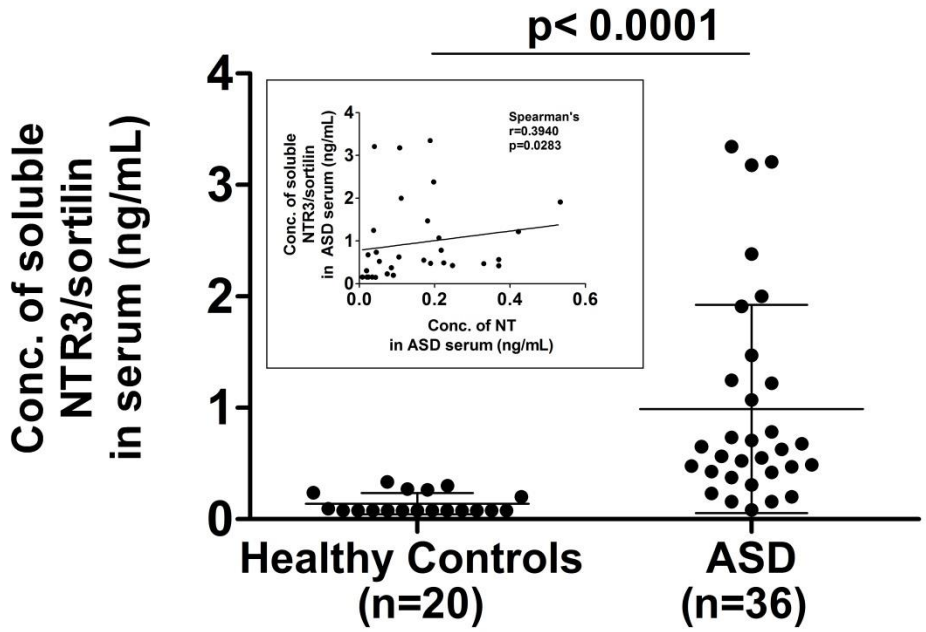


Figure 2.17. Increased serum NTR3/sortilin levels in ASD that positively correlate with serum NT levels. Levels of soluble NTR3/sortilin and circulating NT (inset) were measured by ELISA in the serum of children with ASD, as compared to age- and sex-matched healthy normal controls. Significance of comparisons is denoted by $p<0.0001$. A positive correlation between serum sortilin and NT levels is shown using the Spearman rank correlation test (Spearman's $r=0.3940$, $p=0.0283$).

3.4. Discussion

The impetus for this present study came from our previous reports that serum NT levels are increased in children with ASD (Angelidou, et al., 2010;Tsilioni, et al., 2014). Unlike the previous studies that used murine microglia (Dicou, et al., 2004), the present findings show that NT stimulates primary microglia obtained from human brains and also an immortalized human cell line, microglia-SV40. Human microglia express *only* NTR3/sortilin and not the other known NT receptors, NTR1 or NTR2. NTR3/sortilin was previously shown to be expressed in the immortalized human (Martin, et al., 2003;Martin, et al., 2005) and murine (Dicou, et al., 2004) microglia cell lines. Stimulation with NT (nM) increases gene expression and release of the pro-inflammatory cytokine IL-1beta and chemokines CXCL8, CCL2 and CCL5, relevant to ASD. Increased levels of these mediators have been reported in the brains (Vargas, et al., 2005;Ashwood, et al., 2011) and blood (Masi, et al., 2015;Tsilioni, et al., 2015;Choi, et al., 2016) of patients with ASD.

Stimulation with NT also increases proliferation of human microglia-SV40 only after 48 h, which is in agreement with the previous report that incorporation of ^{3}H-thymidine in human microglia C13NJ in response to NT remains unchanged after 24 h (Martin, et al., 2003). In addition, although the selective NTR1 antagonist SR48692 was previously shown to reduce binding of labeled NT (IC_{50} of 238 ± 46 nM) to this cell line (Martin, et al., 2003), for our study neither the NTR1 nor NTR2 antagonists had any effect on pro-inflammatory cytokine/chemokine release from NT-stimulated human microglia-SV40, implying that these NT responses are mediated via NTR3/sortilin.

The difference in the localization of immunodetectable NTR3/sortilin from the cytosol in non-stimulated microglia to the cell surface after stimulation with NT led us to wonder whether sortilin may be secreted extracellularly. The co-localization of NTR3/sortilin with the ionized calcium binding adaptor molecule-1 (Iba-1), which interacts with actin and is often used to detect microglia (Ito, et al., 1998), allowed us to speculate that Iba1 may participate in movement of NTR3/sortilin to the cell surface. The extracellular domain of NTR3/sortilin, known as soluble sortilin (Navarro, et al., 2002), is shown here to be secreted from NT-stimulated cultured human microglia. Soluble sortilin is also increased in the serum of children with ASD, as compared to normal healthy children. Since there is a strong positive correlation of the soluble receptor with serum NT levels in these same ASD patients, serum NTR3/sortilin may bind to serum NT and limit its biological activity much like the soluble IL-1 receptor binds to circulating IL-1beta (Hannum, et al., 1990).

We further characterized the activation of human microglia-SV40 by numerous other stimuli that could be relevant to the pathophysiology of ASD, including the peptides, corticotropin releasing hormone (CRH) (Tsilioni, et al., 2014) and substance P, as well as the known stimuli which can switch microglia into pro-inflammatory M1 phenotype (Pannell, et al., 2014;Nakagawa and Chiba, 2014;Hong, et al., 2016). Increases in the mediator gene levels were measured by qRT-PCR, while mediator protein release was determined by specific ELISA kits and Western blot analysis, as summarized in Table 2.1. The gene and protein expression of receptors involved in stimulation of human microglia-SV40 in response to these peptides and LPS were also measured (Table 2.1), while those that are unidentified in our present study have been

characterized in murine or other human microglia cell lines (Righi, et al., 1989;Janabi, et al., 1995;Nagai, et al., 2001;Pocock and Kettenmann, 2007).

TABLE 2.1. Characterization of the immortalized cell line of human microglia-SV40 activation

Stimulus	Mediator gene expression	Mediator release	Receptor expression
Neurotensin (NT) (10 or 100 nM)	IL-1β, IL-6, IL-18, IL-33, CXCL8, CCL3, CCL2, CCL5	IL-1α, IL-1β, IL-10, IL12p70, BDNF, CXCL8, CCL2, CCL5, GM-CSF, sTREM1, sNTR3/sortilin	NT receptor 3 /sortilin
Corticotropin-releasing hormone (CRH,100 nM)	IL-1β, IL-6, CXCL8, CCL2, CCL5	IL-1β, CCL2,CCL5, GM-CSF	CRH receptor 1
Substance P (SP, 100 nM)	IL-1β, IL-6, CCL2, CCL5	IL-1β, CCL2	Neurokinin (NK) receptor 1
Interleukin (IL)-33 (1 or 10 ng/mL)	IL-1β, IL-6, CCL2, CCL5	IL-1β, CCL2, TNF	-
Lipopolysaccharide (LPS, 10 or 100 ng/mL)	IL-1β, IL-6, IL-18, IL-33, CCL3, CCL4, CCL5	IL-1β, IL-6, BDNF, CXCL8, CCL2,CCL5, GM-CSF, NGF, TNF	Toll-like receptor 4 (TL4)
Interferon gamma (INF-γ, 10 or 50 U/mL)	IL-1β, IL-6, IL-18, IL-33, CCL3, CCL4, CCL5	IL-1β, IL-6, CXCL8, CCL2, CCL5, TNF	-
ATP (10 or 100 μM)	IL-1β, IL-6, IL-18, IL-33, CCL3, CCL5	IL-1β, IL-6, CXCL8, GM-CSF, NGF, TNF	-

Interleukin (IL); Brain-derived neurotrophic factor (BDNF); Chemokine C-C or C-X-C motif ligand (CCL or CXCL); Granulocyte-macrophage colony-stimulating factor (GM-CSF); Nerve growth factor (NGF); tumor necrosis factor (TNF); soluble triggering receptor expressed on myeloid cells 1 (sTREM1); soluble NT receptor 3/sortilin.

[All conditions were performed in triplicates for each data set and were repeated three times .Mediator gene and protein levels measured for stimulated cells were significantly increased ($p<0.001$ (**) or $p<0.0001$ (***), as compared to non-stimulated control cells]

Our experiments also explored the signaling pathways involved following NT stimulation of human microglia mediated by NTR3/sortilin by the use of a kinase array blot that detects phosphorylated substrates; this array shows increased mTOR signaling, following which it was determined that activation of the mTOR pathway is necessary for pro-inflammatory mediator expression in NT-stimulated human microglia. These

findings provide evidence why some ASD patients with gene mutations in one negative regulatory protein of mTOR, the phosphatase and tensin homolog (PTEN) (Butler, et al., 2005;Varga, et al., 2009), develop inflammation of the brain that is linked to ASD pathogenesis (Costa-Mattioli and Monteggia, 2013;Kassai, et al., 2014).

Our findings also suggest that mTOR signaling is involved in the transcriptional regulation of pro-inflammatory cytokine and chemokine synthesis in human microglia. This effect may be mediated via the activation of nuclear factor-kappa B (NF-κB) (Dan, et al., 2008) and the signal transducer and activator of transcription (STAT) pathways (Saleiro and Platanias, 2015), critical for transcription of pro-inflammatory cytokines and chemokines (Weichhart, et al., 2008). An important new finding is that the flavonoids luteolin and methoxyluteolin significantly inhibit *gene* expression of all the pro-inflammatory mediators, as well as the activation of mTOR, after stimulation by NT.

Not only did the flavonoids inhibit NT-stimulated microglial responses, but they also inhibit LPS-stimulated pro-inflammatory cytokine and chemokine synthesis in microglia, as previously shown in murine microglia (Kao, et al., 2011;Park, et al., 2007). Greater concentrations of flavonoids are required to inhibit cytokine or chemokine protein release, compared to gene expression. This apparent discrepancy may be due to some initial secretion before gene expression is fully inhibited by the lower flavonoid concentrations. Alternatively, there may be differential inhibition of gene expression, involving the inhibition of nuclear transcription targets (NF-κB or STAT), compared to cytokine or chemokine protein secretion. Instead, mediator trafficking and secretion may involve inhibition of specific target proteins involved in vesicle fusion, such as soluble N-

ethylmaleimide-sensitive factor attachment proteins (SNARE complexes) (Yang, et al., 2013;Yang, et al., 2015).

Luteolin had previously been shown to inhibit only LPS-induced IL-6 release from both primary and immortalized (BV-2) murine microglia (Jang, et al., 2008). *In vivo*, luteolin reversed autism-like behavior in the maternal immune activation mouse model (Parker-Athill, et al., 2009;Hsiao, et al., 2012;Garay, et al., 2013). Moreover, two pilot, open-label, clinical studies using a luteolin-containing dietary formulation reported significant improvement in attention and sociability in children with ASD (Theoharides T.C., et al., 2012a;Taliou, et al., 2013). Methoxyluteolin is a more potent inhibitor than luteolin or rapamycin (Li, et al., 2014a) and other mTOR inhibitors (Wander, et al., 2011), indicating that it could be developed into an effective treatment for ASD.

The several new findings presented in this report increase our understanding of the mechanistic pathway by which the peptide NT may play an important causal role in the pathogenesis of ASD. Presently, there is no clinically effective drug to treat the pathophysiology of ASD (Ghosh, et al., 2013). Targeting NTR3/sortilin and/or using methoxyluteolin may provide important novel therapeutic approaches for ASD.

Chapter 3

Neuropeptide Stimulation of TNF, CXCL8 and VEGF Release from Human Mast Cells involves mTOR Activation, Inhibited by Methoxyluteolin

3.1. Introduction

Mast cells (MC) are unique immune cells that derive from hematopoietic precursors (Rodewald, et al., 1996;Chen, et al., 2005a) and mature in vascularized tissues (Williams and Galli, 2000). In addition to stimulation by the high affinity IgE receptor (FcεRI) (Rivera, et al., 2008;Brown, et al., 2008;Galli, 2016), MC are also activated by neurotensin (NT) (Lazarus, et al., 1977a;Lazarus, et al., 1977b;Kulka, et al., 2007) and substance P (SP) (Fewtrell, et al., 1982;Theoharides, et al., 2010). Stimulated MC release pre-stored molecules, such as histamine, tryptase, lipid-derived mediators (Boyce, 2007), as well as *de novo* synthesized pro-inflammatory mediators, such as TNF, CXCL8 and vascular endothelial growth factor (VEGF) (Theoharides, et al., 2012b). MC are not only involved in allergies (Wedemeyer, et al., 2000;Galli and Tsai, 2012;Modena, et al., 2016), immunity (Galli, et al., 2005;Galli, et al., 2008a) and mast cell disorders, including mastocytosis (Theoharides, et al., 2015d), but are also implicated in inflammatory processes (Lawrence, et al., 2002;Theoharides, et al., 2015d).

Elevated circulating levels of NT have been reported in patients with skin disorders, such as psoriasis and atopic dermatitis (Vasiadi M, et al., 2012), as well as in children with autism spectrum disorders (ASD) (Angelidou, et al., 2010;Tsilioni, et al., 2014). Increased levels of SP have been associated with psoriasis (Remröd, et al., 2007) and other inflammatory diseases (O'Connor, et al., 2004;Nicoletti, et al., 2012). MC-derived CXCL8 enhances recruitment of immune cells to the site of inflammation (Salamon, et al., 2005), while VEGF can increase vascular permeability (Donelan, et al., 2006), disrupting both the gut-blood barrier (Wallon, et al., 2008;Theoharides and Doyle, 2008) and the blood-brain barrier (Theoharides, 1990;Esposito, et al., 2002;Ribatti,

2015). Even though the allergic stimulation of MC is fairly well characterized (Rivera, et al., 2008), less is known about the non-allergic stimulation of MC (Yu, et al., 2016). A receptor was recently identified as being critical in pseudo-allergies, some of which are due to MC activation by cationic drugs (McNeil, et al., 2015), but NT and SP were not studied.

The phosphatidylinositol-3-kinase (PI3K)-dependent mammalian target of rapamycin (mTOR) kinase pathway (Dibble and Cantley, 2015) has been implicated in the regulation of normal MC homeostasis and function (Kim, et al., 2008a), including FcεRI-mediated allergic responses (Smrz, et al., 2011). mTOR serves as the catalytic subunit in two complexes: (a) mTORC1, involved in protein translational control by phosphorylation of ribosomal S6 kinases (S6K) and the eukaryotic initiation factor 4E-binding proteins (4EBP1), and (b) mTORC2 implicated in cytoskeleton reorganization (Laplante and Sabatini, 2012). Downregulation of the upstream mTOR inhibitory protein phosphatase and tensin homolog (PTEN) leads to increased MC activation (Furumoto, et al., 2006) and a mastocytosis-like state (Furumoto, et al., 2011). Other studies have revealed that mTORC2 mediates MC chemotaxis (Kuehn, et al., 2011;Halova, et al., 2012) and proliferation of neoplastic human MC (Smrz, et al., 2011).

There are still no clinically effective MC inhibitors. Disodium cromoglycate (cromolyn) inhibits rodent peritoneal MC histamine release (Theoharides, et al., 1980), but it does not effectively inhibit either murine MC (Oka, et al., 2012) or human (Weng, et al., 2012) MC. Moreover, the flavonoid quercetin has been shown to be more potent than cromolyn in inhibiting photosensitivity in humans (Weng, et al., 2012).Even though the local application of a cromolyn ointment reduced histamine-induced pruritus in

human skin, it apparently did so via modulation of sensory nerves and not by inhibiting MC (Vieira Dos, et al., 2010).

Hence, there is an urgent need for developing effective inhibitors of human MC. Luteolin (3',4',5,7-tetrahydroxyflavone) is a natural anti-oxidant and anti-inflammatory flavonoid, which also inhibits MC release of histamine, TNF and VEGF (Kempuraj, et al., 2005), as well as leukotrienes and prostaglandin D2 (Kimata, et al., 2000b). We recently showed that its structural analog, 3',4',5,7-tetramethoxyflavone (methoxyluteolin) is a more potent MC inhibitor (Weng, et al., 2015), and is also metabolically more stable (Walle, 2007).

In the present study, we report that mTOR activation is involved in the stimulation of human MC by NT and SP and that this mechanism is inhibited by the flavonoids luteolin and methoxyluteolin.

3.2 Materials and Methods

Materials

SP (S6883) and NT (N6383) were obtained from Sigma-Aldrich (St Louis, MO). PI3K inhibitor LY294002 (Cell Signaling Technology) and mTOR inhibitors (rapamycin and Torin 1 (TOCRIS biosciences, Bristol, UK) were purchased. The flavonoids luteolin and methoxyluteolin were obtained from Pharmascience Nutrients (Clear Water, FL). RNeasy Mini (Qiagen Inc., Valencia, CA) and iScript cDNA synthesis kits (BioRad, Hercules, CA) were purchased. Taqman gene expression primers/assays for TNF (Hs99999043_m1), CXCL8 (Hs00174103_m1), VEGFA (Hs00900055_m1) and GAPDH endogenous control (4310884E) were purchased from Applied Biosystems (Foster City, CA). ELISA kits for TNF (DY210), CXCL8 (DY208) and VEGF (DY293B) were purchased from R&D Biosystems (Minneapolis, MN). Rabbit mAb for mTOR (7C10), pmTORSer2448 (D9C2), pmTORSer2481, mTORC1 substrates p70S6K (49D7), pp70SK Thr389 (108D2), 4EBP1 (53H11) and p4EBP1Thr37/46 (236B4), as well as the loading control β-actin (D6A8) were purchased from Cell Signaling Technology (Beverly, MA).

Methods

Human MC Culture

Human LAD2 MC (kindly supplied by Dr. A.S. Kirshenbaum, National Institutes of Health, NIH), derived from human MC leukemia (Kirshenbaum, et al., 2003;Sismanopoulos N, et al., 2012), were cultured in StemPro®-34 SFM medium (Invitrogen, Carlsbad, CA) supplemented with 100 U/ml penicillin/streptomycin and 100

ng/mL recombinant human stem cell factor (rhSCF, kindly supplied by Biovitrum AB, Stockholm, Sweden). These cells have been used numerous times in our laboratory and shown to behave like primary human umbilical-cord blood derived MC. Cell viability was determined by trypan blue (0.4 %) exclusion.

LAD2 MC Treatments

LAD2 MC were stimulated with NT (1-10 µM, Sigma-Aldrich, St. Louis, MO) or SP (1 µM, Sigma-Aldrich) and/or pre-incubated with DMSO or the following inhibitors: (a) PI3K inhibitors (LY294002 (LY), 1-50 µM, 2h, Cell Signaling Technology), (b) mTOR inhibitors (rapamycin (Rap), 0.005-0.2 µM or Torin1, 0.005-0.2 µM TOCRIS biosciences, Bristol, UK) and (c) the flavonoids (Lut or MethLut, 1-50 µM, 2,12 or 24 h, PharmaScience Nutrients, Clearwater, FL). All inhibitors were dissolved in water or DMSO with final concentration of < 0.1 %.

Pro-inflammatory Mediator Release

Mediator release in cell-conditioned culture medium/ supernatant fluid was determined by using commercial available ELISA kits (R&D Systems, Minneapolis, MN) as per manufacturer's instructions. LAD2 MC (1×10^5 cells/well) were seeded in 96-well flat-bottom Falcon Culture plates (Becton Dickinson) prior to pretreatment with inhibitors/flavonoids (for 30 mins) and SP or NT-stimulation for 24 h. MC supernatant fluids were collected and TNF, CXCL8 and VEGF mediator release was measured. For all experiments, the control cells were treated with equal volume of culture medium or DMSO, and the minimum detectable level for all by ELISA was 5 pg/mL.

MC Degranulation

LAD2 MC were stimulated with the neuropeptides, substance P (SP 1 µM, Sigma-Aldrich) or NT (10 µM, Sigma-Aldrich) for 30 min. Beta-hexosaminidase release was assayed using a fluorometric method as previously reported (Zhang, et al., 2011). Briefly, beta-hexosaminidase activity in the supernatant fluid and cell lysates (0.5×10^5 cells/tube, were lysed with 1% Triton X-100 to measure residual cell-associated beta-hexosaminidase) were incubated with substrate solution (p-nitrophenyl-N-acetyl-beta-D-glucosaminide from Sigma-Aldrich) in 0.1 M NaOH/0.2 M glycine. Absorbance was read at 405 nm in a plate reader (Lab Systems Multiskan RC, Thermo Fisher), and the results were expressed as percentage of beta-hexosaminidase released over the total.

Pro-inflammatory Mediator Gene Expression

Total RNA from MC and microglia was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription (RT) was performed with 300 ng of total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Gene expression analysis of TNF, CXCL8 and VEGF mediators in LAD2 MC (2.5×10^5 cells/6-well plate), those preincubated with inhibitors/flavonoids and/or those stimulated with SP or NT, was measured after 6 h. Quantitative real-time PCR (qRT-PCR) was performed using Taqman gene expression assays to assess the gene expression of mediators using validated oligonucleotide primers (Applied Biosystems, Carlsbad, CA). Samples were run at 45 cycles using Applied Biosystems 7300 Real-Time PCR System. Relative mRNA abundance was determined from standard curves run for each experiment. Gene expression was normalized to GAPDH endogenous control.

Assessing mTOR Activation by Western blot & ELISA

The activation of mTOR was assessed by phosphorylation (P) of downstream mTORC1 and mTORC2 substrates by Western blot analysis and using Pathscan phospho-ELISA kits (R&D Systems). LAD2 MC (2×10^6 cells/T-25 flask) were pretreated with inhibitors or flavonoids for the indicated doses and times, the stimulated with NT or SP for 0-60 mins, before cells were pelleted and lysates harvested in RIPA buffer (Sigma Aldrich), containing Halt Protease and Phosphatase Inhibitor Cocktails (Thermo Fisher Sci., Rockford, IL). The total protein concentrations were determined by the bicinchoninic acid assay (Thermo Fisher Sci.) using bovine serum albumin protein as standards. The total proteins (20 or 40 μg) were separated using 4-20 % Mini-PROTEAN TGX™ precast gels (BioRad) under SDS denaturing conditions and electro-transferred onto PVDF membranes (EMD Millipore). Blocking was carried out with 5% (w/v) BSA in Tris-buffered saline (TBS) containing 0.1 % Tween-20. The membranes were probed with the following primary antibodies: mTOR (7C10), P-mTOR (Ser2448), P-mTOR (Ser2481), mTORC1 substrates p70S6K, P-p70SK (Thr389; Ser371), 4EBP1 and P-4EBP1 (Thr-37/46), and β-actin as the loading control (Cell Signaling Technology/ CST, Danvers, MA). All proteins were visualized with horseradish peroxidase-conjugated secondary antibodies and then by SuperSignal West Pico enhanced chemiluminescence (Thermo Fisher Sci). To quantitate changes in protein phosphorylation, the films were scanned and densitometric analysis will be carried out using Image J software. In parallel experiments, using the CST's PathScan mTOR, P-mTOR (Ser2448), p70S6K, P-p70S6K (Thr389), 4EBP1 and P-4EBP1 sandwich ELISA kits, the levels of total and phosphorylated mTORC1 substrates were measured in MC after treatments described.

Statistical Analysis

All conditions were performed in triplicate and all experiments were repeated at least three times (n=3). Results from cultured cells are presented as mean ± SD. Comparisons were made between (1) control and stimulated cells using the unpaired 2-tailed, Student's *t*-test, with significance of comparisons denoted by the horizontal lines and by $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***). Multiple comparisons were also made between (1) for all conditions with stimulated cells and with inhibitors/flavonoids using one-way ANOVA, followed by post-hoc analysis by Dunnett's Multiple Comparison Test; significance is denoted by horizontal lines and indicated values $p<0.001$ or $p<0.0001$ and (2) all the inhibitors/flavonoids among themselves using one-way ANOVA, followed by post-hoc analysis by Tukey's Multiple Comparison Test; those conditions for which there is significance denoted by the horizontal brackets and by the corresponding $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***). The analysis was performed by using the GraphPad Prism version 5.0 software (GraphPad Software, San Diego, CA, USA). Representative images for western blots were scanned and analyzed using Image J (NIH, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2015).

3.3. Results

Neuropeptide-stimulated human MC pro-inflammatory mediator release is inhibited by PI3K or mTOR inhibitors

Initially, we investigated the optimal concentrations of NT or SP (1-10 μM) that stimulated the *de novo* pro-inflammatory mediator release from cultured human LAD2 MC. Stimulation by NT (10 μM) and SP (1 μM) significantly increased the release of TNF, CXCL8 and VEGF from human LAD2 MC after 24 h (data not shown). Using these optimal concentrations, we next evaluated the involvement of mTOR signaling by employing pharmacological inhibitors of mTOR, which included the bacterial macrolide and more selective mTORC1 inhibitor rapamycin and the more potent ATP-competitive dual inhibitor of mTOR, Torin1, as well as the upstream PI3K inhibitor LY294002. Pre-treatment of LAD2 MC (24 h) with the inhibitors of mTOR signaling, rapamycin and Torin1, dose-dependently (0.05-0.2 μM) decreases SP-stimulated TNF (Fig. 3.1A) and CXCL8 (Fig. 3.1B) release from human MC. At the optimal inhibitory concentration of 0.2 μM, Torin1 significantly decreases ($p<0.001$) pro-inflammatory mediator release from SP-stimulated LAD2 MC, as compared to rapamycin that is more selective for mTORC1 inhibition. Pre-treatment with the PI3K inhibitor, LY294002 (1-50 μM, 2 h) decreases ($p<0.0001$) TNF and CXCL8 release, with optimal inhibitory concentration of 50 μM (Fig. 3.1C).

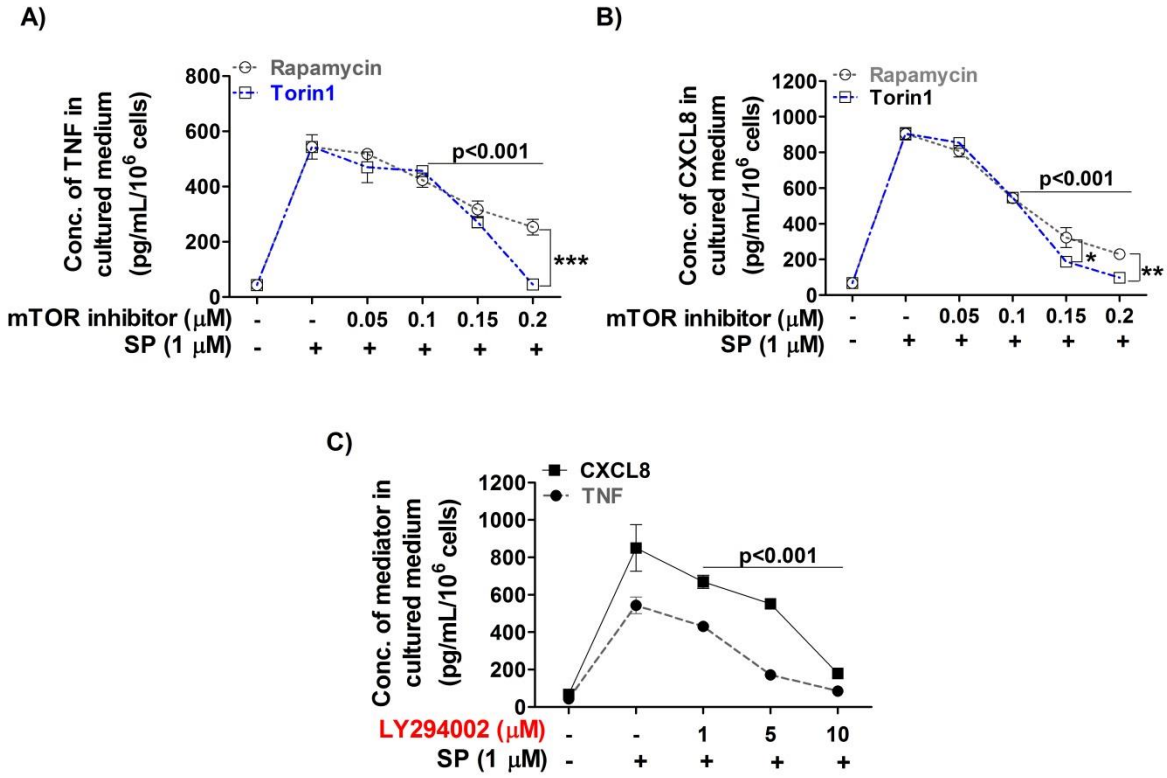


Figure 3.1. Inhibitors of mTORC1 signaling dose-dependently decrease SP stimulated pro-inflammatory mediator release from human MC. LAD2 MC ($0.5x10^6$ cells) were pre-treated with the mTOR inhibitor, rapamycin (0.05-0.2 μM, 24 h), the ATP-competitive dual mTOR inhibitor, Torin1 (0.05-0.2 μM, 24 h) prior to stimulation with SP (1 μM) for 24 h to measure release of (A) TNF and (B) CXCL8 by ELISA. LAD2 MC ($0.5x10^6$ cells) were also pre-treated with the upstream (C) PI3K inhibitor LY294002 (1-50 μM, 2 h), prior to stimulation with SP (1 μM) for 24 h to measure release of TNF and CXCL8 mediators. All conditions were performed in triplicates for each data set and were repeated three times (n=3). Significance of comparisons were made for stimulated cells and those with inhibitors, as denoted by the horizontal lines ($p<0.001$) and also among each of the inhibitors treatments shown by the vertical brackets and by corresponding $p<0.001$ (**) and $p<0.0001$ (***).

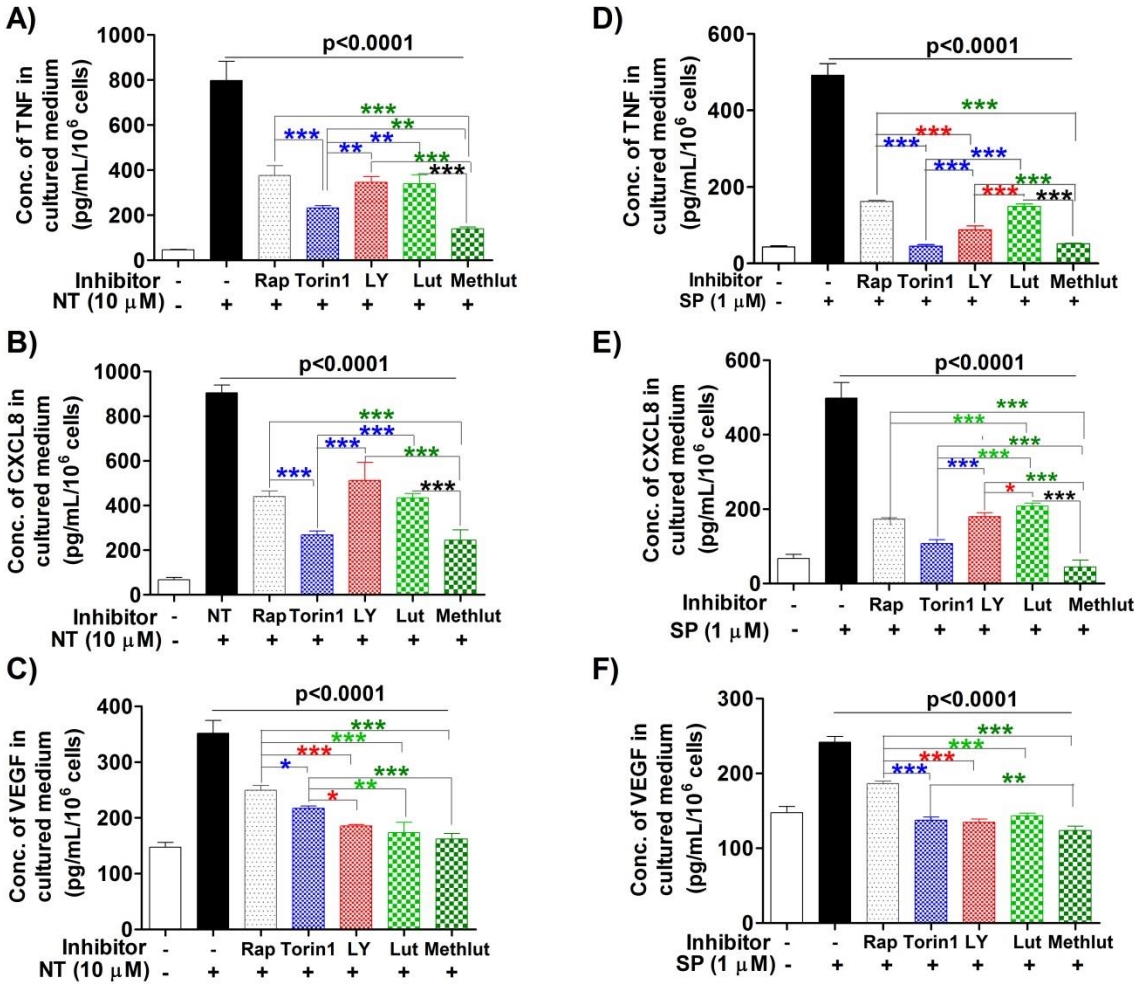


Figure 3.2. Human MC pro-inflammatory mediator release in response to NT or SP is attenuated by PI3K/mTOR inhibitors, and luteolin and methoxyluteolin. LAD2 MC (1 x10^5 cells) were pre-treated with the mTOR inhibitors [rapamycin (Rap) and Torin1, 0.2 μM, 24 h] or the upstream PI3K inhibitor [LY294002, (LY) 50 μM, 2 h] or the natural flavonoids [luteolin (Lut) and methoxyluteolin (Methlut), 50 μM, 30 min], then stimulated with NT (10 μM) (A-C) or the positive control trigger SP (1 μM) (D-F) for 24 h to measure release of TNF, CXCL8 and VEGF mediators by specific enzyme-linked immunosorbent assays (ELISA). All conditions were performed in triplicates for each data set and were repeated three times (n=3). Significance of comparisons were made for stimulated cells and those with inhibitors/flavonoids, as denoted by the horizontal lines ($p<0.0001$) and also among each of the inhibitors/flavonoids treatments shown by the horizontal brackets and by corresponding $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).

Neuropeptide-stimulated human MC mediator release is inhibited by the flavonoids luteolin and methoxyluteolin

We further compared the effects of the PI3K/mTOR inhibitors to the flavonoids luteolin and methoxyluteolin on LAD2 MC mediator release after neuropeptide stimulation for 24 h. LAD2 MC were pre-treated with inhibitors of mTOR (rapamycin or Torin 1, 0.2 μM, 24 h) or PI3K (50 μM, 2h) and the flavonoids luteolin or methoxyluteolin (50 μM, 2 h), and then stimulated by NT (10 μM) or SP (1 μM) for 24 h. The release of TNF, CXCL8 and VEGF significantly decreases in the presence of the flavonoids ($p<0.0001$), after stimulation by either NT (Fig. 3.2 A-C) or SP (Fig. 3.2 D-F). LAD2 MC were first pre-treated with luteolin and methoxyluteolin at various doses (1-50 μM, 2 h), then stimulated by NT (10 μM) for 24 h to identify the most effective concentration inhibiting release of pro-inflammatory mediators. Methoxyluteolin more potently than luteolin ($p<0.001$) decreases the release of all mediators at equimolar flavonoid concentrations (Fig. 3.3).

Neuropeptide-stimulated human MC degranulation is decreased only by the PI3K inhibitor and the flavonoids luteolin and methoxyluteolin

Unlike their effect on *de novo* synthesized mediator release, pre-treatment of LAD2 MC with the mTOR inhibitors (rapamycin or Torin1, 0.2 μM, 24 h), followed by stimulation with either NT (Fig. 3.4A) or SP (Fig. 3.4B) for 30 min have no effect on β-hexosaminidase release from LAD2 MC. Instead, the upstream PI3K inhibitor (LY294002, 50 μM, 2 h), and luteolin and methoxyluteolin (50 μM, 24 h) significantly decrease ($p<0.001$), β-hexosaminidase release (Fig. 3.4).

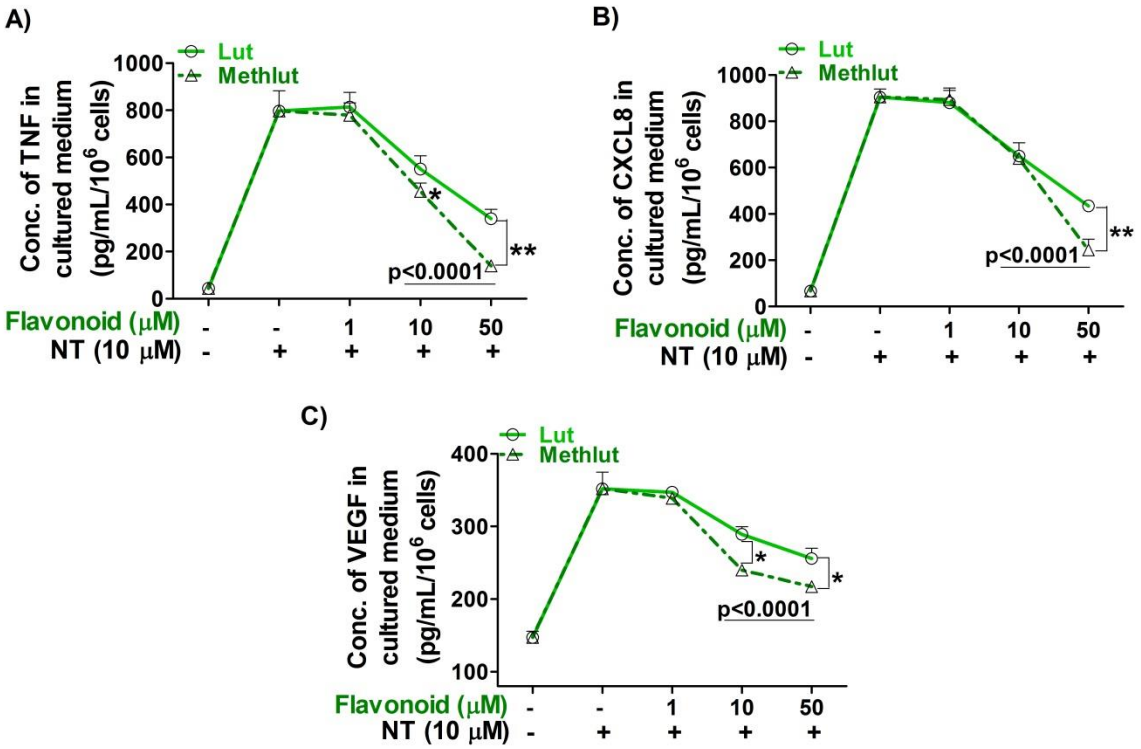


Figure 3.3. Methoxyluteolin more potently than luteolin inhibits NT-stimulated pro-inflammatory mediator release from human MC. LAD2 MC (0.5 x10^6 cells) were pre-treated the flavonoids [luteolin (Lut) and methoxyluteolin (Methlut), 1-50 μM] for 30 min, then stimulated with NT (10 μM) for 24 h to measure release of (A) TNF, (B) CXCL8 and (C) VEGF by ELISA. All inhibitors were dissolved in water or DMSO with final concentration < 0.1 %. All conditions were performed in triplicates for each data set and were repeated three times (n=3). Significance of comparisons were made for stimulated cells and those with flavonoids, as denoted by the horizontal lines ($p<0.0001$) and also among each of the inhibitors treatments shown by the vertical brackets and by corresponding $p<0.05$ (*) and $p<0.001$ (**).

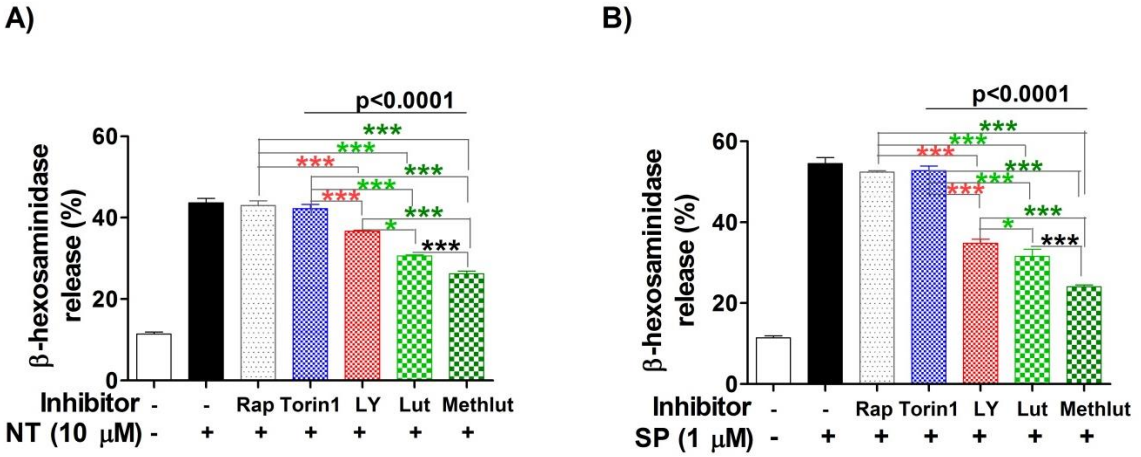


Figure 3.4. Human MC degranulation in response to NT or SP is decreased by the PI3K inhibitor LY294002, luteolin and methoxyluteolin. LAD2 MC ($0.5x10^6$ cells) were pre-treated with the mTOR inhibitors [rapamycin (Rap) and Torin1, 0.2 μM, 24 h], the upstream PI3K inhibitor [LY294002 (LY), 50 μM, 2 h) or the natural flavonoids [luteolin (Lut), methoxyluteolin (Methlut), 50 μM, 2 h), then stimulated with A) NT (10 μM) or B) the positive control trigger SP (1 μM) for 30 min. β-hexosaminidase release was measured to assess pre-stored mediator release via degranulation. All inhibitors were dissolved in water or DMSO with final concentration < 0.1 %. All conditions were performed in triplicates for each data set and were repeated three times (n=3). Significance of comparisons were made for stimulated cells and those with inhibitors/flavonoids, as denoted by the horizontal lines ($p<0.0001$) and also among each of the inhibitors/flavonoids treatments shown by the horizontal brackets and by corresponding $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).

Neuropeptide-stimulated pro-inflammatory mediator gene expression in human MC is decreased by the PI3K or mTOR inhibitors, and luteolin and methoxyluteolin

We further investigated whether mTOR signaling is involved in the synthesis of pro-inflammatory mediators in human MC stimulated by either NT or SP. LAD2 MC were pretreated with the mTOR inhibitors rapamycin and Torin1 (0.2 μM, 24 h) and the upstream PI3K inhibitor LY294002 (50 μM, 2 h), prior to stimulation with NT (10 μM) or SP (1 μM) for 6 h.

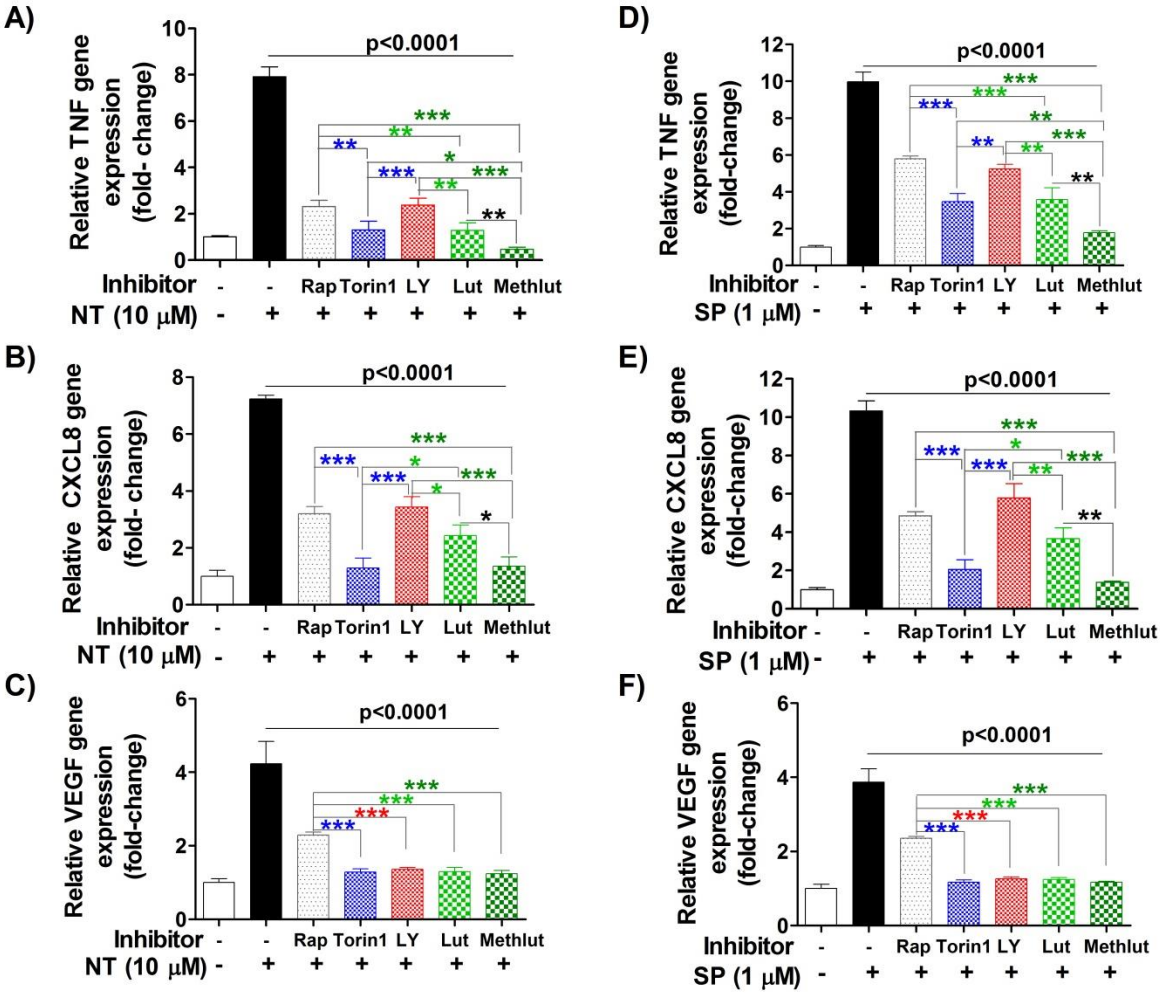


Figure 3.5. Human LAD2 MC pro-inflammatory mediator gene expression in response to NT or SP is inhibited by the PI3K/mTOR inhibitors, luteolin and methoxyluteolin. LAD2 MC ($0.5x10^6$ cells) were pre-treated with the mTOR inhibitors [rapamycin (Rap) and Torin1, 0.2 μM, 2 or 24 h] or the upstream PI3K inhibitor [LY294002 (LY), 50 μM, 2 h] or the natural flavonoids [luteolin (Lut) and methoxyluteolin (Methlut) 50 μM, 30 mins], prior to stimulation with NT (10 μM) **(**A, C and E**)** or the positive control trigger SP (1 μM) **(**B, D and F**)** for 6 h to measure gene expression of TNF, CXCL8 and VEGF by RT-qPCR. All conditions were performed in triplicates for each data set and were repeated three times (n=3). Significance of comparisons were made for stimulated cells and those with inhibitors/flavonoids, as denoted by the horizontal lines ($p<0.0001$) and also among each of the inhibitors/flavonoids treatments shown by the horizontal brackets and by corresponding $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).

Stimulation of LAD2 MC by either NT (Fig. 3.5 A-C) or SP (Fig. 3.5 D-F) significantly increases ($p<0.001$) the gene expression of TNF, CXCL8 and VEGF, which decreases ($p<0.0001$) after treatment with PI3K/mTOR inhibitors (Fig. 3.5).

Pre-treatment with either luteolin or methoxyluteolin also significantly decreases ($p<0.0001$) gene expression of TNF, CXCL8 and VEGF in response to NT or SP (Fig. 3.5). Methoxyluteolin (50 µM) is more potent ($p<0.05$) than luteolin or the PI3K inhibitor LY294002 ($p<0.001$) for inhibition of pro-inflammatory mediator gene expression (Fig. 3.5).

Neuropeptide stimulation of human MC activates signaling via mTORC1 that is inhibited by methoxyluteolin

To investigate the specific mTOR complex activated in response to NT or SP stimulation, Western blot analysis was performed to detect the total and phosphorylated (p) levels of mTOR (pmTORSer2448, an indicator of signaling via mTORC2) and the mTORC1 substrates p70S6K (pp70S6KThr389) and 4EBP1 (p4EBP1Thr37/46) proteins **(**Fig. 3.6A). Densitometric analysis revealed that stimulation of LAD2 MC with either NT (10 µM) or SP (1 µM) increases ($p<0.0001$) the levels of pmTORSer2448 (Fig. 3.6B and 3.6C) and the downstream mTORC1 substrate, pp70S6KThr389 (Fig. 3.6D and 3.6E) after 20 min, when compared to control cells. Noteworthy, stimulation with NT or SP had no effect on the levels of p4EBP1Thr37/46.

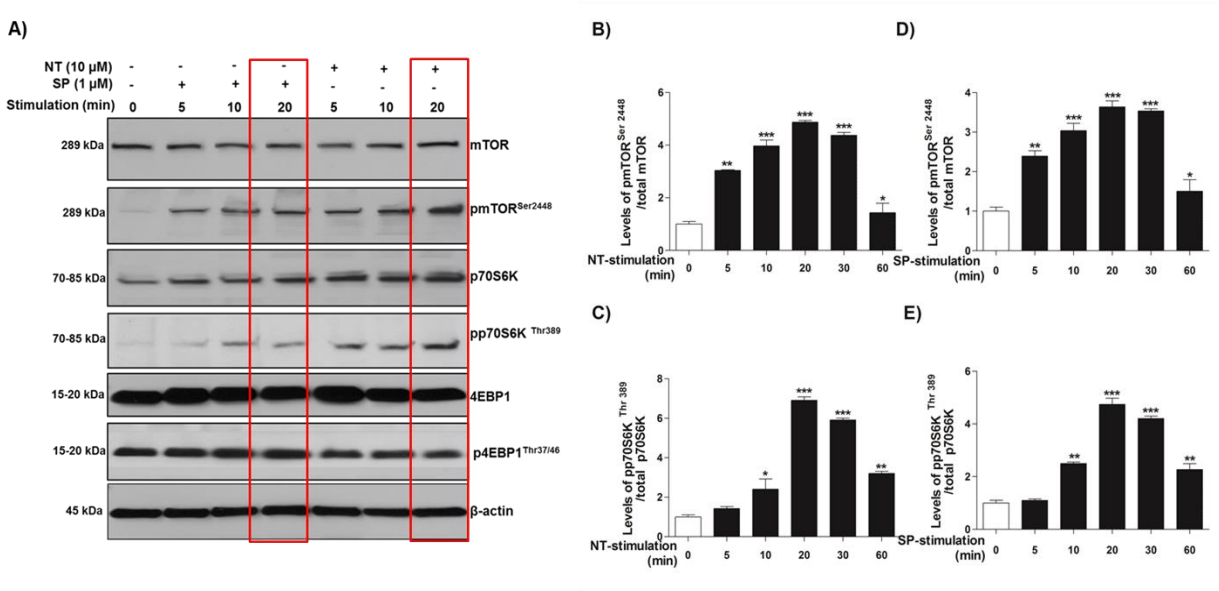


Figure 3.6. NT and SP stimulate mTOR activation in human LAD2 MC. LAD2 MC ($1x10^6$ cells) were stimulated with the positive control trigger SP (1 µM) or NT (10 µM) for 0-60 mins to probe for the total and phosphorylated levels of mTOR, and substrates p70S6K and 4EBP1 by Western blot analysis; peak mTOR activation is denoted at 20 min for pmTORSer2448 and pp70S6KThr389 proteins **(A)**. Results were quantified using densitometric analyses for pmTORSer2448 to mTOR levels and pp70S6KThr389 to p70S6K levels in response to NT (10 µM) (B, D) or SP (1 µM) (C, E). All conditions were performed in triplicates for each data set, repeated three times (n=3) and results presented as mean ± SD. Significance of comparisons is denoted by $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).

We further evaluated the effects of luteolin and methoxyluteolin on mTOR signaling in NT or SP stimulated LAD2 MC by comparing their inhibition to those treated with the PI3K or mTOR inhibitors. LAD2 MC were pre-incubated with rapamycin or Torin 1 (0.2 µM, 24 h) or LY294002 (50 µM, 2 h) or luteolin and methoxyluteolin (50 µM, 2 h) prior to NT (10 µM) (Fig. 3.7A) or SP (1 µM) (Fig. 3.7B) stimulation for 20 min. The PI3K/mTOR and the flavonoids inhibitors significantly decrease levels of pmTORSer2448 and pp70S6KThr389, compared to those of neuropeptide-stimulated LAD2 MC.

To further quantify the levels of phosphorylated mTOR and it substrates, phospho-ELISAs were also performed on LAD2 MC pre-treated with the PI3K/mTOR inhibitors or luteolin and methoxyluteolin, prior to stimulation with NT or SP. Levels of pmTORSer2448 (Fig. 3.7 C and F) and pp70S6KThr389 proteins (Fig. 3.7 D and G) increase in response to stimulation with NT or SP, but significantly decrease ($p<0.001$) after pre-treatment with all the inhibitors and flavonoids. Noteworthy, methoxyluteolin shows greater reduction of pmTORSer2448 and pp70S6KThr389 levels, when compared to the PI3K inhibitor LY294002 ($p<0.0001$) or luteolin at equimolar flavonoid concentrations ($p<0.001$) (Fig. 3.7).

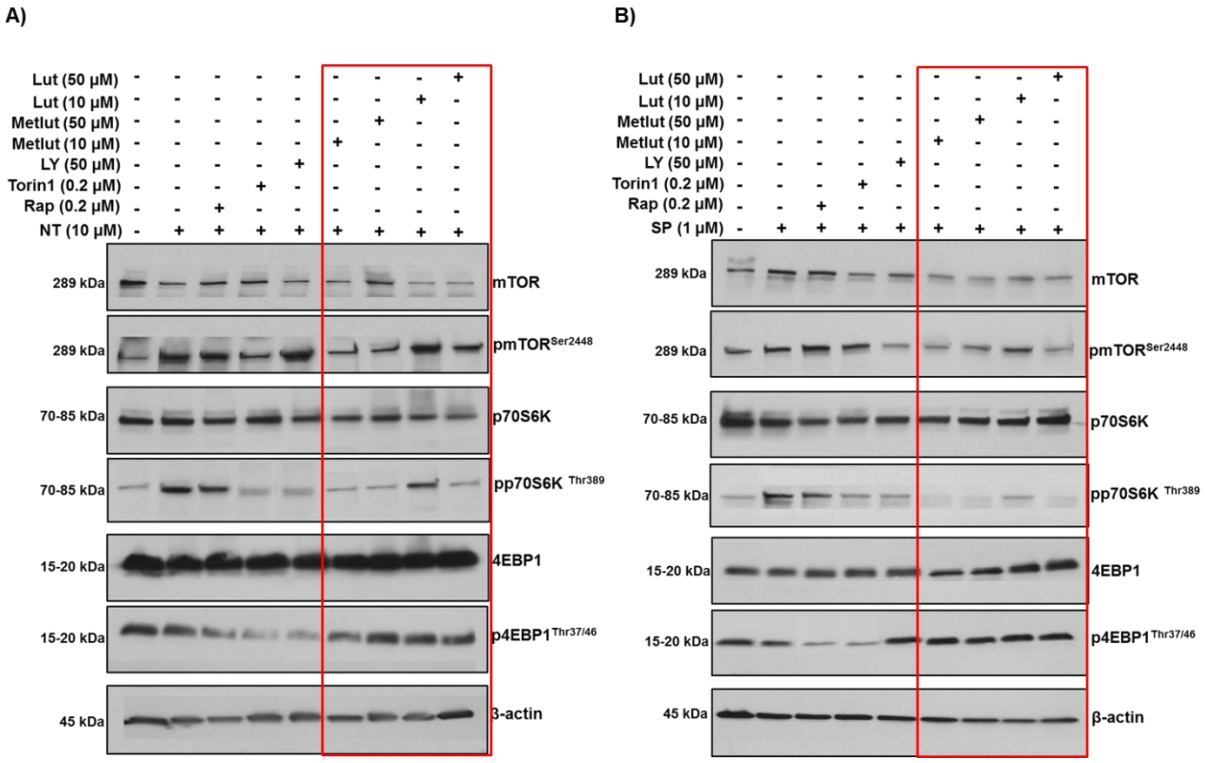
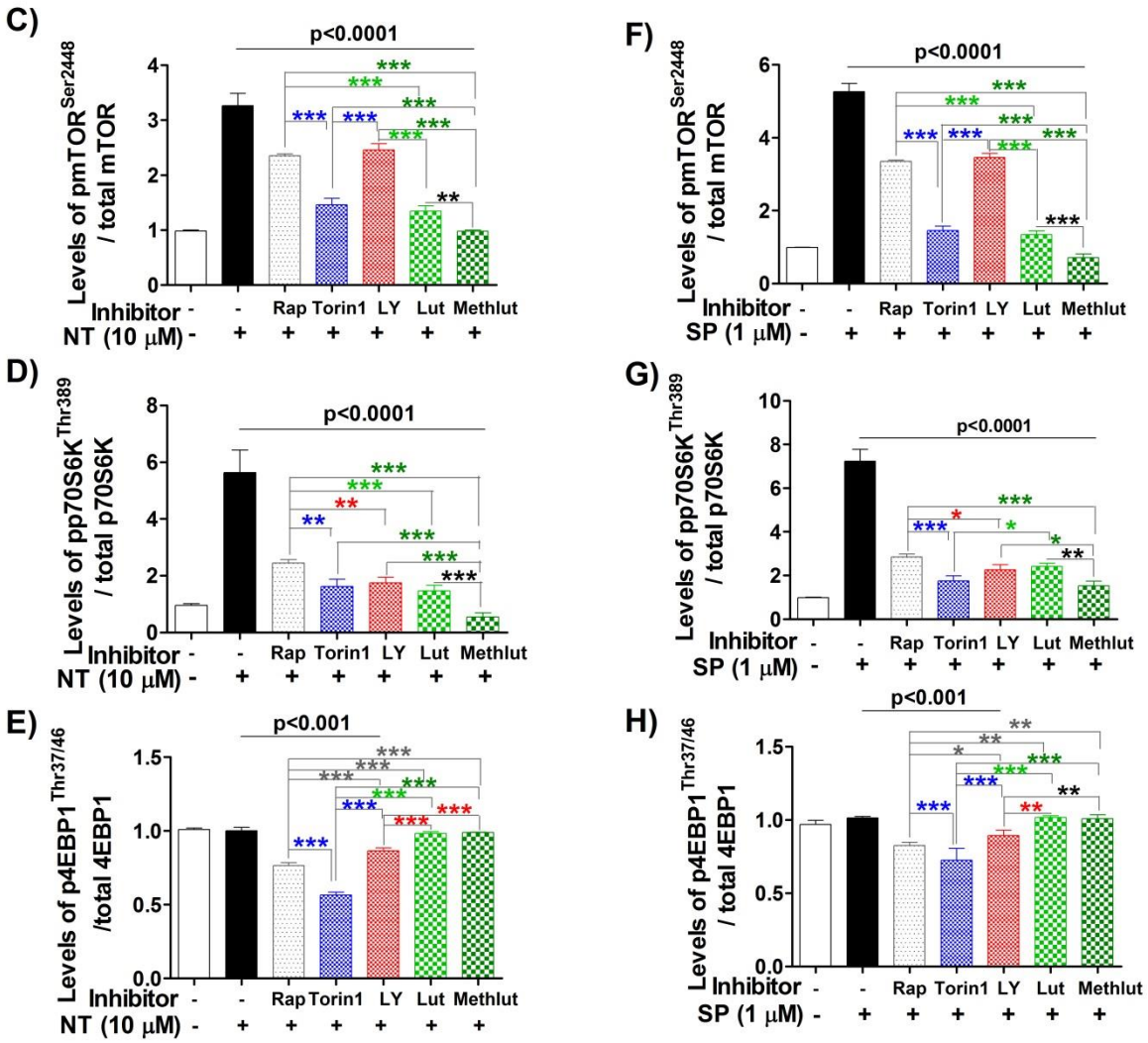


Figure 3.7. NT or SPstimulated mTOR activation in LAD2 MC is inhibitable by luteolin and methoxyluteolin. LAD2 MC ($1x10^6$ cells) were pre-treated with the mTOR inhibitors [rapamycin (Rap) and Torin1, 0.2 µM, 24 h] or the upstream PI3K inhibitor [LY294002 (LY), 50 µM, 2 h] or the natural flavonoids [luteolin (Lut) and methoxyluteolin (Methlut), 10 or 50 µM, 30 mins], prior to stimulation with (A) NT (10

μM) or (B) SP (1 μM) for 20 min to probe for the total and phosphorylated levels of mTOR, and substrates p70S6K and 4EBP1 by Western blot analysis.



The protein levels of mTOR and pmTOR Ser2448 (C, F) and the downstream mTORC1 substrates, p70S6K and pp70S6K Thr389 (D, G) and, 4EBP1 and p4EBP1 Thr37/46 (E, H) were measured using specific total or phospho-ELISA kits for equal amounts of protein lysates, with ratios of phosphorylated to total proteins normalized. All conditions were performed in triplicates for each data set and were repeated three times (n=3). Significance of comparisons were made for stimulated cells and those with inhibitors/flavonoids, as denoted by the horizontal lines ($p<0.001$ or $p<0.0001$) and also among each of the inhibitors/flavonoids treatments shown by the horizontal brackets and by corresponding $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).

3.4. Discussion

A novel finding reported here is the involvement of mTOR signaling in the neuropeptide stimulation of human MC for the synthesis and release of TNF, CXCL8 and VEGF. Stimulation of human LAD2 MC by either NT or SP markedly elevates levels of pmTORSer2448, the mTOR site phosphorylated by upstream PI3K signaling and the mTORC1-dependent p70S6K Thr389 site, indicating activation of mTORC1. Both the gene expression and release of *de novo* synthesized TNF, VEGF and CXCL8 decreases in the presence of pharmacological inhibition of PI3K (LY294002) or mTOR (Torin1>>rapamycin) signaling. It had been reported that the neoplastic human MC lines, such as LAD2 MC have increased mTOR expression compared to primary MC (Smrz, et al., 2011), with activated mTORC1 critical for the survival of MC (Smrz, et al., 2011). Signaling via mTOR had previously been implicated in FcεRI-mediated allergic cytokine release (Smrz, et al., 2011) and in the regulation of normal MC homeostasis (Kim, et al., 2008a). Our results are also in agreement with the previous report that rapamycin blocks FcεRI-mediated PI3K-dependent activation of mTORC1 signaling and *de novo* synthesized IL-6 and CXCL8 release from murine MC (Kim, et al., 2008b).

An important new finding reported here is that the flavonoids luteolin and methoxyluteolin significantly inhibit *gene* expression and release of all the pro-inflammatory mediators and the activation of mTORC1. Our findings suggest that mTOR signaling is involved in the transcriptional regulation of mediator induction in human MC after neuropeptide stimulation, possibly through inhibition of nuclear factor-kappa B (NF-κB) (Dan, et al., 2008) and the signal transducer and activator of transcription

(STAT) (Laplante and Sabatini, 2013;Saleiro and Platanias, 2015), both critical for transcription of pro-inflammatory cytokines and chemokines (Weichhart, et al., 2008). It was previously shown that luteolin and methoxyluteolin can inhibit TNF synthesis and release by inhibiting NF-κB activation (Weng, et al., 2015). In fact, PI3K/mTOR, NF-κB and STAT have been implicated in FcεRI-mediated allergic activation of human MC (Rivera, et al., 2008;Kim, et al., 2008b;Blatt, et al., 2012;Siegel, et al., 2013), while rapamycin was previously shown to inhibit TNF gene expression in rat MC (Park, et al., 2012).

It is critical to note that unlike the mTOR inhibitors, luteolin and methoxyluteolin also inhibits MC degranulation. A possible explanation for this is that the flavonoids target multiple signaling proteins involved in pro-inflammatory mediator gene expression, synthesis and release: (a) gene expression via PI3K, mTORC1 (Kim, et al., 2008b) and nuclear transcription targets (NF-κB or STAT) (Dan, et al., 2008;Laplante and Sabatini, 2013;Saleiro and Platanias, 2015), (b) mediator production via mTORC1 involved in protein translation (Dibble and Cantley, 2015;Howell and Manning, 2011) and (c) mediator secretion via mTORC2 for cytoskeleton rearrangement (Laplante and Sabatini, 2012), and (d) specific target proteins involved in vesicle fusion, such as soluble N-ethylmaleimide-sensitive factor attachment proteins (SNARE complexes) (Yang, et al., 2013;Yang, et al., 2015). Instead, the preferential mTORC1 inhibitor, rapamycin and the dual mTORC1/mTORC2 inhibitor, Torin 1 (Thoreen, et al., 2009) did not inhibit NT or SP-stimulated human MC degranulation, which is in agreement with the recent report showing that FcεRI-mediated allergic MC degranulation is regulated by the rictor protein alone, independent of mTORC2 signaling (Smrz, et al., 2014).

The present findings may have additional importance in pathophysiological settings. For instance, activation of human MC via FcεRI up-regulates the surface expression of the SP neurokinin receptors (Kulka, et al., 2007), while the SP-related peptide hemokinin A that binds to neurokinin 1 receptor further augments the allergic stimulation of MC (Sumpter, et al., 2015). We had shown that SP (Asadi, et al., 2012) and NT (Alysandratos, et al., 2012) induce the expression of corticotropin-releasing hormone (CRH) receptor-1 in human MC, through which NT synergistically with CRH stimulates VEGF release (Donelan, et al., 2006). MC express the NT receptor 1 and synthesize precursor NT peptides (Cochrane, et al., 2011). MC can also degrade NT (Piliponsky, et al., 2008), indicating tight regulation.

The increased levels of NT in patients with ASD (Angelidou, et al., 2010;Tsilioni, et al., 2014) and its ability to stimulate mTOR activation in MC reported her is additionally intriguing. Communication between MC and microglia has been invoked in inflammation of the brain (Skaper, et al., 2012;Skaper, et al., 2014a) and increasing evidence indicates that MC and inflammation of the brain are invoked in the pathogenesis of ASD (Theoharides, et al., 2016b). Microglia, the resident immune cells of the brain are stimulated by MC-derived histamine (Dong, et al., 2014a) and tryptase (Zhang, et al., 2012b), and are implicated in the pathogenesis of ASD (Vargas, et al., 2005;Morgan, et al., 2010;Rodriguez and Kern, 2011;Morgan, et al., 2014;Gupta, et al., 2014). Moreover, about 1-5% of ASD cases have gene mutations in regulatory proteins, including PTEN, permitting uninhibited mTOR activation (Willsey and State MW, 2015;Lee, 2015).

Understanding the regulation and inhibition of MC stimulation especially by non-allergic stimuli is important. Our present findings of mTOR activation in neuropeptide induced MC stimulation and its inhibition by methoxyluteolin has clear therapeutic potential. In fact, two open-label clinical studies have reported that a luteolin-containing dietary formulation significantly improved attention and sociability in children with ASD (Theoharides T.C., et al., 2012a;Taliou, et al., 2013). Methoxyluteolin could be superior not only because it is more potent inhibitor of MC and mTOR activation, but is metabolically stable (Walle, 2007) allowing for greater absorption and movement into the brain than luteolin, due its increased methylation.

Chapter 4

Regulation of Human Mast Cell Degranulation and *De novo* Synthesized Mediator Release by the Cytoskeletal Ezrin and Moesin

4.1. Introduction

Mast Cells (MC) function as effectors of both innate and adaptive immune responses by participating in host defense, immunological tolerance (Galli, et al., 2005;Galli, et al., 2008a;Tsai, et al., 2011), allergic reactions (Rivera, et al., 2008;Brown, et al., 2008;Galli, 2016) and inflammation (Lawrence, et al., 2002;Theoharides, et al., 2015d). MC are involved in allergic diseases via stimulation by the high affinity IgE receptor (FcεRI) (Rivera, et al., 2008;Brown, et al., 2008;Galli, 2016) and are also activated by peptides, such as such substance P (SP) (Fewtrell, et al., 1982;Theoharides, et al., 2010a)via binding to the SP neurokinin receptors (Kulka, et al., 2007), which belong to the G-protein coupled receptor family (GPCRs) (Chahdi, et al., 1998;Barrocas, et al., 1999) or the direct modulation of G-proteins (Mousli, et al., 1994;Chahdi, et al., 2000;Palomaki and Laitinen, 2006).

MC can induce allergic and inflammatory responses via the differential (Theoharides, et al., 1982) or selective (Kandere-Grzybowska, et al., 2003) secretion of *de novo* synthesized mediators via vesicular release (40-80 nM). These include a plethora of the pro-inflammatory mediators, such as the cytokines tumor necrosis factor (TNF) and vascular endothelial growth factor (VEGF) or the chemokine (C-C motif) ligand 2 (CCL2) (Theoharides, et al., 1982;Theoharides and Cochrane, 2004;Wedemeyer, et al., 2000). In addition, stimulation of MC can lead to the immediate secretion of the pre-formed mediators histamine, tryptase, tumor necrosis factor (TNF) and proteoglycans that are stored within cytoplasmic granules (800–1000 nm), unrelated to the *de novo* synthesis (Theoharides, et al., 2007b)via MC degranulation or exocytosis (Pang and Sudhof, 2010).

Granule trafficking to the plasma membrane for MC degranulation requires the coordinated rearrangement of the filamentous (F)-actin cytoskeleton (Tolarova, et al., 2004), while granule fusion requires the elevated levels of intracellular calcium (Douglas and Kagayama, 1977;Weng, et al., 2015), calcium-binding proteins (Sagi-Eisenberg, 2007) and phosphatidylinositol-4,5-bisphosphate (PIP_2) (Jahn, 2004;Wollman and Meyer, 2012) or transmembrane protein/receptors, prior to exocytosis of granular contents mediated by the soluble N-ethylmaleimide-sensitive factor attachment proteins (SNARE complexes) (Puri, et al., 2003;Puri and Roche, 2008;Woska, Jr. and Gillespie, 2011). This process had been described for both hormonal secretion from the pituitary gland (Nagasawa, et al., 1970) and for the MC (Uvnas, 1963;Lagunoff and Benditt, 1960;Douglas, 1978).

The ezrin, radixin and moesin protein complex (ERM) serve as intramolecular scaffolds that cross-link F-actin to PIP_2 on the plasma membrane (Algrain, et al., 1993;Bretscher, 1999;Tsukita and Yonemura, 1999;Tsujita and Itoh, 2015) in a various cell types, thereby regulating processes such as cell morphology, motility, signaling and trafficking (Bretscher, et al., 2002;Fehon, et al., 2010;Neisch and Fehon, 2011). In the dormant state, the ERM domains involved in binding to F-actin and PIP_2 form inactive monomers or dimers (Gary and Bretscher, 1993;Gary and Bretscher, 1995;Tsukita, et al., 1997). Activation of ERM requires the sequential binding of PIP_2 to two sites releasing an auto-inhibitory linker (Barret, et al., 2000;Ben-Aissa, et al., 2012) and the subsequent phosphorylation on a conserved Thr residue in the actin-binding domain of ERM (pezrinThr567, pradixinThr564 and pmoesinThr558) (Matsui, et al., 1998;Nakamura, et al., 1999;Fievet, et al., 2004;Ben-Aissa, et al., 2012).

This ERM phosphorylation is evident in neurons (Lamb, et al., 1997;Haas, et al., 2007;Paglini, et al., 1998;Jeon, et al., 2010), microglia (Moon, et al., 2011;Kashimoto, et al., 2013), fibroblasts(Shaw, et al., 1998), epithelial cells (Bretscher, 1983;Tachibana, et al., 2015;Hatano, et al., 2013), endothelial cells (Koss, et al., 2006;Lee, et al., 2010), B-lymphocytes (Pore and Gupta, 2015;Pore, et al., 2016), T-lymphocytes (Ivetic and Ridley, 2004;Shaffer, et al., 2009;Charrin and Alcover, 2006), natural killer cells(Helander, et al., 1996), monocytes/macrophages(Iontcheva, et al., 2004;Zawawi, et al., 2010), platelets (Nakamura, et al., 1999), insulin-secreting β-cells (Lopez, et al., 2010) and in MC (Sieghart, et al., 1978;Wells and Mann, 1983;Wang, et al., 1999;Theoharides, et al., 2000;Correia, et al., 1996;Olson, et al., 2009;Halova, et al., 2013;Staser, et al., 2013).

ERM are suspected targets of several kinases downstream of the phosphatidylinositol-3-kinase (PI3K)-dependent mammalian target of rapamycin (mTOR) kinase pathway (Dibble and Cantley, 2015) that exists in two complexes: (a) mTORC1, involved in protein translational control and MC homeostasis (Kim, et al., 2008a) (b) mTORC2 implicated in cytoskeleton reorganization (Laplante and Sabatini, 2012) and MC chemotaxis (Kuehn, et al., 2011;Halova, et al., 2012). These kinases include the kinase C (PKC) isoform θ(Pietromonaco, et al., 1998;Kempuraj, et al., 2005)and the Rho family of GTPases (RhoA/Rac/Cdc42) (Hirao, et al., 1996;Mackay, et al., 1997;Haas, et al., 2007;Liu, et al., 2010;Gulhati, et al., 2011;Oh and Jacinto, 2011). The Rho family have emerged has key regulators of the actin cytoskeleton (Hall, 2012) and have been reported to regulate MC exocytosis (Brown, et al., 1998;Azouz, et al., 2012;Higashio, et al., 2016).

To date, little is known about the specific roles of ERM proteins in the regulation of MC cytoskeleton for both degranulation and vesicular release. Previously, our laboratory had identified moesin as the 78-kDa phosphoprotein in the rat MC (Sieghart, et al., 1978) and its specific inhibitory Ser/Thr phosphorylation sites on FERM after pre-treatment with the MC inhibitor, disodium cromoglycate (cromolyn) (Correia, et al., 1996;Wang, et al., 1999) and flavonoids (Theoharides, et al., 2000). The PKC isoform θ had been shown to phosphorylate these inhibitory sites on moesin in the presence of flavonoids (Kempuraj, et al., 2005). It was proposed that phosphorylation of the sites other than Thr558 may have the opposite effect leading to the inhibition of secretion. Moesin apparently is not required for MC motility in moesin-deficient mice (Doi, et al., 1999), but these animals are prone to increased alveolar fibrosis, indicating an impairment in MC activation and wound healing (Tellechea, et al., 2016;Douaiher, et al., 2014).

In the present study, we investigate the importance of moesin and other ERM in human MC activation in response to either IgE-mediated allergic or SP-mediated non-allergic stimulation. Our findings reveal that, while ezrin phosphorylation is mainly implicated in the regulation of MC degranulation and *de novo* synthesized mediator release, activation of moesin is diminished in the presence of PI3K/mTOR inhibitors or the flavonoids luteolin and methoxyluteolin, indicating its regulatory function for MC activation.

4.2. Materials and Methods

Materials

Recombinant human stem cell factor (rhSCF) was kindly donated by Biobitrum AB, (Stockholm, Sweden). Human IgE was obtained from Millipore (Billerica, MA). Anti-IgE was purchased from Life technologies (Carlsbad, CA). SP and NT were obtained from Sigma-Aldrich (St Louis, MO). PI3K inhibitors (wortmannin and LY294002 (Cell Signaling Technology), mTOR inhibitors (rapamycin and Torin 1 (TOCRIS biosciences, Bristol, UK) were purchased. The flavonoids luteolin and methoxyluteolin were obtained from Pharmascience Nutrients (Clear Water, FL). Ambion Silence® Select siRNAs targeting human MSN (#1:s8984 & #2: s8986) and EZN (s14795), as well as scramble non-targeting siRNA controls (#1 & #2) were obtained from Life Technologies (Grand Island, NY). RNeasy Mini (Qiagen Inc., Valencia, CA) and iScript cDNA synthesis kits (BioRad, Hercules, CA) were purchased. Taqman gene expression primers/assays for moesin/MSN (Hs00741306_m1), ezrin/EZN(Hs00931653_m1), radixin/RDX (Hs00988414_g1), TNF (Hs99999043_m1), CCL2 (Hs00234140_m1), VEGF (Hs00900055_m1) and GAPDH endogenous control (4310884E) were purchased from Applied Biosystems (Foster City, CA). ELISA kits for TNF (DY210),VEGF (DY293B) and CCL2 (DY279) were purchased from R&D Biosystems (Minneapolis, MN). Goat pAb for moesin (C-15) and pmoesinThr 558 (sc-12895) were purchased from Santa Cruz Biotechnology Inc. (Dallas, Texas). Rabbit mAb for mTOR (7C10), pmTORSer2448 (D9C2), pmTORSer2481, mTORC1 substrates p70S6K (49D7), pp70SK Thr389 (108D2), 4EBP1 (53H11) and p4EBP1Thr37/46

(236B4), as well as the loading control β-actin (D6A8) were purchased from Cell Signaling Technology (Beverly, MA).

Methods

Human MC Culture

LAD2 MC (kindly provided by Dr. A.S. Kirshenbaum, National Institutes of Health, NIH) were derived from a single patient with human MC leukemia as reported previously (Kirshenbaum, et al., 2003;Sismanopoulos N, et al., 2012). LAD2 MC were cultured in StemPro®-34 SFM medium (Invitrogen, Carlsbad, CA) supplemented with 100 U/mL penicillin/streptomycin and 100 ng/mL recombinant human stem cell factor (rhSCF, kindly supplied by Sweden Orphan Biovitrum AB, Stockholm, Sweden). These cells have been used numerous times in our laboratory and shown to behave like primary human umbilical-cord blood derived MC. Cell viability was determined by trypan blue (0.4 %) exclusion test.

Gene silencing by siRNA

LAD2 MC (1×10^6 cells) were transfected with two different siRNAs (pre-designed and validated Silencer® Select siRNAs #1 and #2) targeting either MSN or both MSN and EZN to downregulate endogenous protein, as well as scramble siRNA controls (Life Technologies, Grand Island, NY). siRNA (10-100 nM) transfection was carried out using Lipofectamine™ RNAiMAX in Opti-MEM® reduced serum and antibiotic-free medium (Life Technologies) for 48 or 72 h according to manufacturer's instructions, prior to evaluation of gene knockdown quantitative real-time PCR (qRT-PCR). Cell viability was done by trypan blue (0.4 %) exclusion test.

Gene Expression by quantitative real time PCR (qRT-PCR)

LAD2 MC (5×10^5 cells) were treated the same way as described above with various drugs before stimulation with either SP (2 μM) or anti-IgE (10 μg/mL) for 6 h. Total RNA was extracted with an RNeasy Mini kit (Qiagen Inc., Valencia, CA) in accordance with the manufacturer's specifications. An iScript cDNA synthesis kit (BioRad, Hercules, CA) was used for reverse-transcription of each sample. Quantitative RT-PCR was performed using Taqman gene expression assays (Applied Biosystems, Foster City, CA) for MSN, EZN, RDX, TNF, CCL2 and VEGF. Samples were run at 45 cycles using a real-time PCR system (7300, Applied Biosystems). Relative mRNA expression was determined from standard curves run with each experiment. Gene expression was normalized to GAPDH endogenous control.

Cellular inhibitor treatments

LAD2 MC (1×10^6 cells) were stimulated with NT (10 μM, Sigma-Aldrich, St. Louis, MO) or SP (2 μM, Sigma-Aldrich) and/or pre-incubated with DMSO or the following inhibitors: (a) PI3K inhibitors (LY294002 (LY), 50 μM, 2h, Cell Signaling Technology), (b) mTOR inhibitors (rapamycin (Rap), 0.2 μM or Torin1, 0.2 μM TOCRIS biosciences, Bristol, UK) and (c) the flavonoids (Lut or MethLut, 50 μM, 2,12 or 24 h, PharmaScience Nutrients, Clearwater, FL). All inhibitors were dissolved in water or DMSO with final concentration of < 0.1 %.

MC Degranulation by β-Hexosaminidase Release

LAD2 MC (0.5×10^5 cells) were primed overnight with human myeloma IgE (2 μg/mL, Chemicon International, Temecula, CA) and stimulated by anti-human IgE (10

μg/ml, Invitrogen) for 30 min to induce allergic stimulation. LAD2 MC were also stimulated by the neuropeptides NT (10 μM) or SP (2 μM, Sigma-Aldrich) for 30 mins. β-hexosaminidase release was assayed using a fluorometric method as previously reported (Zhang, et al., 2011). Briefly, β-hexosaminidase activity in the supernatant fluid and cell lysates (0.5 x 10^5 cells/tube, were lysed with 1% Triton X-100 to measure residual cell-associated β-hexosaminidase) were incubated with substrate solution (p-nitrophenyl-N-acetyl-beta-D-glucosaminide from Sigma-Aldrich) in 0.1 M NaOH/0.2 M glycine. Absorbance was read at 405 nm in a plate reader (Lab Systems Multiskan RC, Thermo Fisher), and the results were expressed as percentage of β-hexosaminidase released over the total.

Pro-Inflammatory mediator release by ELISA

Mediator release in cell-conditioned culture medium/ supernatant fluid was determined by using commercial available ELISA kits (R&D Systems, Minneapolis, MN) as per manufacturer's instructions. MC (1×10^5 cells/well) were seeded in 96-well flat bottom Falcon cell culture plates (Becton Dickinson, Franklin Lakes, NJ) with SP (2 μM) or NT (10 μM) in triplicates for 24 h. Supernatant fluids were collected and TNF, VEGF and CCL2 release was measured. For all experiments, the control cells were treated with equal volume of culture medium only. The minimum detectable level for all mediators by ELISA was 5 pg/mL.

Activation of mTOR and Moesin by Western blot

The active conformation of moesin in stimulated LAD2 MC was assessed by probing for phosphorylation of moesin, while mTOR activation was determined by phosphorylation of mTOR and substrates p70S6K and 4EBP1. LAD2 MC (2×10^6 cells/condition) were lysed using protein lysis radio-immuno precipitation (RIPA) buffer (Sigma- Aldrich, St. Louis, MO) in the presence of Halt Protease and Phosphatase Inhibitor Cocktails (Thermo Fisher Sci., Rockford, IL), after stimulation with NT (10 µM) or SP (2 µM) for 0-60 min. The total protein concentrations were determined by the bicinchoninic acid assay (Thermo Fisher Sci.) using bovine serum albumin protein (BSA) as standards. The total cellular proteins (20 or 40 µg) were separated using 4-20 % Mini-PROTEAN TGX™ precast gels (BioRad, Hercules, CA) under SDS denaturing conditions and electro-transferred onto PVDF membranes (Millipore, Carrigtwohill, Ireland). Blocking was carried out with 5% (w/v) BSA in Tris-buffered saline (TBS) containing 0.1 % Tween-20. The membranes were probed with the following primary antibodies: moesin, phospho-moesin (Thr558), mTOR (7C10), phospho-mTOR (Ser2448), phospho-mTOR (Ser2481), mTORC1 substrates p70S6K, phospho-p70SK (Thr389; Ser371), 4EBP1 and phospho-4EBP1 (Thr-37/46), as well as beta-actin for the loading control (Cell Signaling Technology). All proteins were visualized with horseradish peroxidase-conjugated secondary antibodies and then by enhanced chemiluminescence (SuperSignal West Pico, Thermo Fisher Sci). To quantitate changes in protein phosphorylation, the films were scanned and densitometric analysis will be carried out using Image J software.

Statistical Analysis

All conditions were performed in triplicate and all experiments were repeated at least three times (n=3). Results from cultured MC are presented as mean ± SD. Comparisons were made between (1) control and stimulated cells ; (2) stimulated cells with and without an inhibitor; (3) inhibitory effects of methoxyluteolin and luteolin using the unpaired 2-tailed, Student's *t*-test. Significance of comparisons is denoted by $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).The analysis was performed by using the GraphPad Prism version 5.0 software (GraphPad Software, San Diego, CA, USA).

4.3. Results

Efficiency of siRNA-induced downregulation of moesin and ezrin in human MC

Previous studies have reported that human MC pre-dominantly express moesin and ezrin of the ERM proteins, while only low levels of radixin is detectable (Doi, et al., 1999). Hence, to investigate the importance of moesin, and distinguish its function from ezrin, in the regulation of human MC mediator release, two different validated MSN siRNAs #1 or #2 (50 nM) targeting moesin and an EZN siRNA (50 nM) for ezrin were used to induce either downregulation of moesin or moesin and ezrin levels in LAD2 MC. Gene expression analysis by real-time quantitative PCR (qRT-PCR) revealed >95% knockdown of moesin in siRNA-transfected MC after 48 or 72 h, compared with scramble (Sc#1 and #2) siRNA transfected or control LAD2 MC (Fig. 4.1A). While the gene levels of ezrin remain unchanged in LAD2 MC with downregulated moesin levels, those in double MSN/EZN siRNA transfected LAD2 MC decrease by ~40 % (Fig. 4.1B). Intriguingly, gene levels of radixin in the double MSN/EZN siRNA transfected LAD MC increase by two-fold, indicating some compensatory radixin increase (Fig. 4.1C).

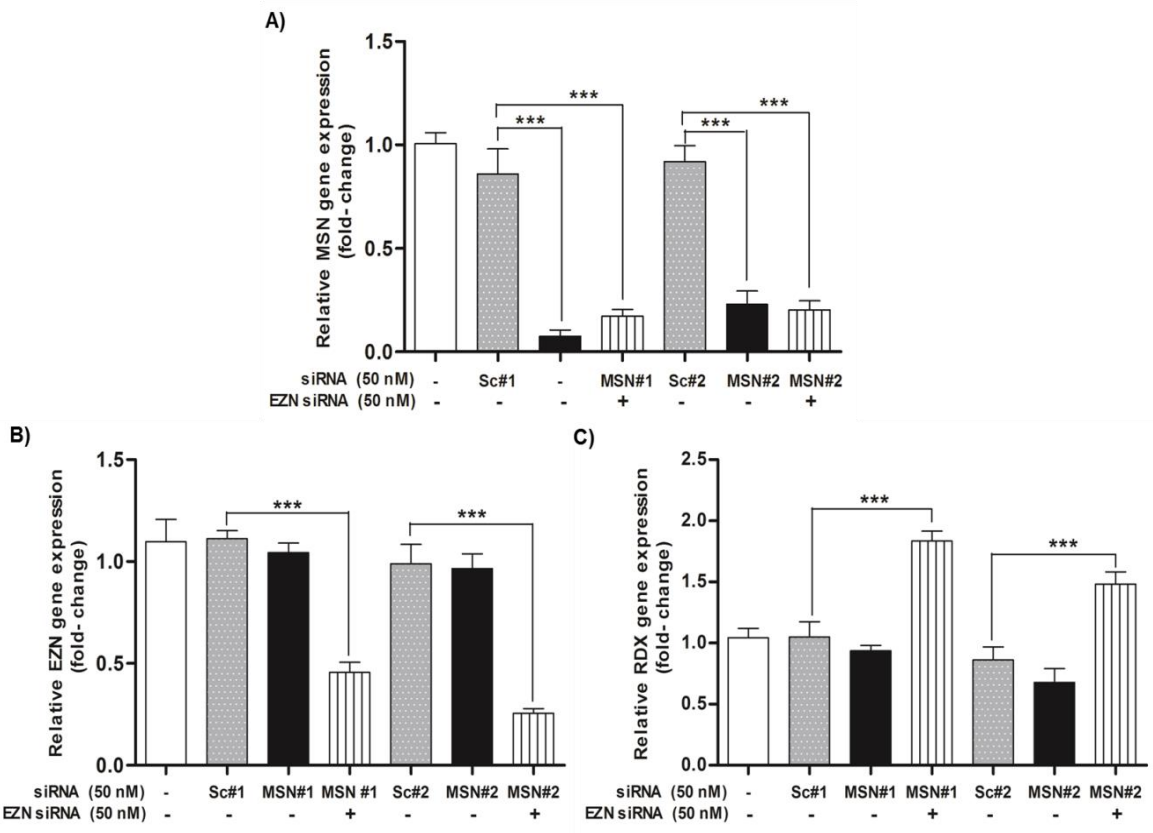


Figure 4.1. Efficiency of siRNA induced single- or double-knockdown of moesin and ezrin in LAD2 MC. LAD2 MC ($1x10^6$ cells) were transfected with two different siRNAs targeting MSN #1 & #2 or the combination of two different siRNAs targeting MSN and EZN, as well as scramble siRNA (Sc) #1 & #2 at concentrations of 50 nM for 72 h. Cells were harvested and analyzed for gene expression by qRT-PCR to determine transfection efficiency and moesin or ezrin gene silencing by measuring gene levels of (A) MSN and (B) EZN. Gene levels of (C) RDX were assessed to determine any compensatory increases in radixin, as part of the ERM. All conditions were performed in triplicates for each data set, repeated three times (n=3) and results presented as mean ± SD. Significance of comparisons is denoted by $p<0.0001$ (***) for each pairwise comparison.

Human MC degranulation in response to either allergic or neuropeptide stimulation is decreased in with downregulated moesin and ezrin gene levels

To evaluate the importance of moesin and ezrin in the secretion of pre-formed mediators from human MC, β-hexosaminidase release was assayed to measure MC degranulation in response to the allergic stimulus IgE/anti-IgE or the peptide SP. LAD2 MC pre-treated with MSN and EZN siRNAs and with significant downregulation of moesin and ezrin levels (after 72 h) had significantly decreased ($p<0.001$) release of β-hexosaminidase, compared with scramble (Sc#1 and #2) siRNA transfected or control LAD2 MC after stimulation by IgE/anti-IgE **(**Fig. 4.2A) or SP (Fig. 4.2B) for 30 mins.

Surprisingly, secretion of β-hexosaminidase from LAD2 MC pre-treated with MSN siRNA alone and with significant down-regulated moesin levels (data not shown) was comparable to scramble (Sc#1 and #2) siRNA transfected or control LAD2 MC after allergic or neuropeptide stimulation **(**Fig. 4.2).

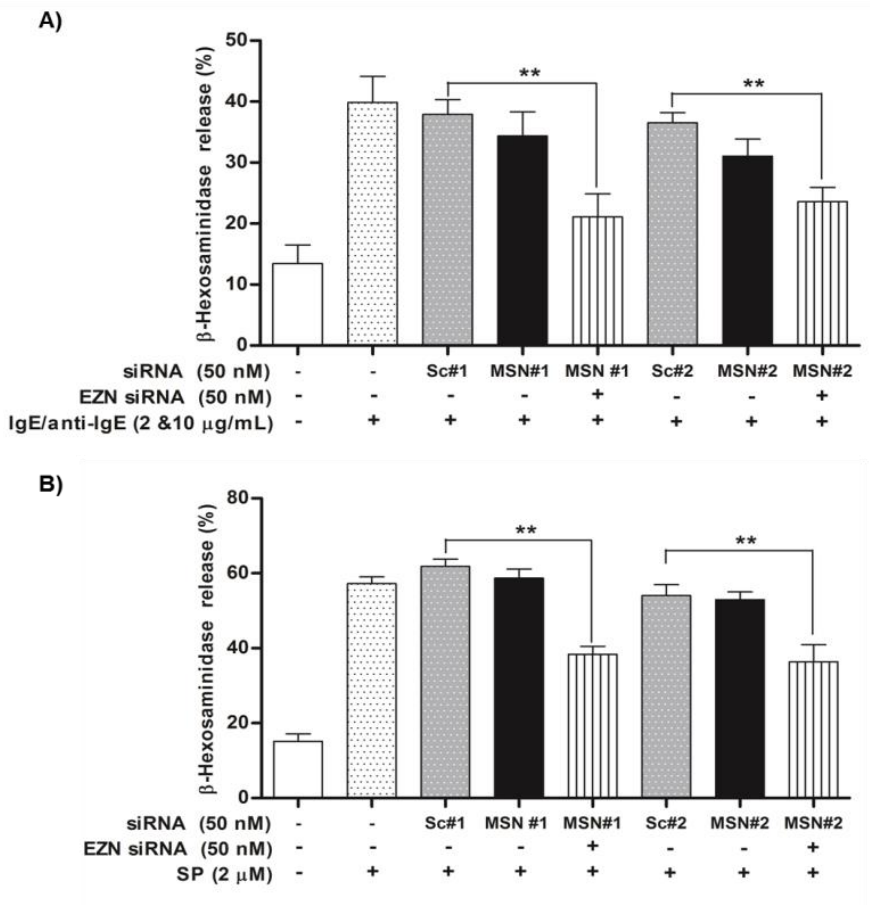


Figure 4.2. The effect of MSN & EZN siRNA induced knockdown on human MC degranulation. LAD2 MC transfected with siRNAs targeting MSN (#1&#2) and/or EZN or the scramble siRNA (Sc) #1&#2 controls as described in Fig. 1.1, ($0.5x10^6$ cells) were stimulated with (A) IgE/anti-IgE (2 or 10 μ/mL) or (B) SP (2 μM) for 30 min. β-hexosaminidase secretion was measured to assess pre-stored mediator release via degranulation. All conditions were performed in triplicates for each data set, repeated three times (n=3) and results presented as mean ± SD. Significance of comparisons is denoted by $p<0.001$ (**) for each pairwise comparison.

Pro-inflammatory mediator release from human MC with downregulated moesin and ezrin is decreased in response to either allergic or neuropeptide stimulation

To determine whether moesin and ezrin are implicated in the regulation of *de novo* synthesized mediator release from human MC, enzyme-linked immunosorbent assays (ELISAs) were performed. Pretreatment of LAD2 MC with MSN and EZN siRNAs, which resulted in LAD2 MC with significantly downregulated moesin and ezrin, released significantly less ($p<0.001$) TNF,VEGF and CCL2 after stimulation with IgE/anti-IgE for 24 h when compared to those from stimulated scramble (Sc#1 and #2) siRNA treated LAD2 MC (Fig. 4.3).

Similarly, pretreatment of LAD2 MC with significantly downregulated moesin and ezrin, prior to stimulation by SP resulted in significantly less ($p<0.001$) release of all three pro-inflammatory mediators, compared to those from stimulated -scramble (Sc#1 and #2) siRNA LAD2 MC (Fig. 4.4). Noteworthy, stimulation of LAD2 MC by either IgE/anti-IgE with significantly downregulated ezrin and moesin levels, resulted in significantly less ($p<0.001$) release of TNF,VEGF and CCL2, when compared to those from stimulated LAD2 MC with down-regulated moesin alone, but normal ezrin levels, suggesting that ezrin primarily regulates human MC pro-inflammatory mediator release (and may compensate for the function of moesin).

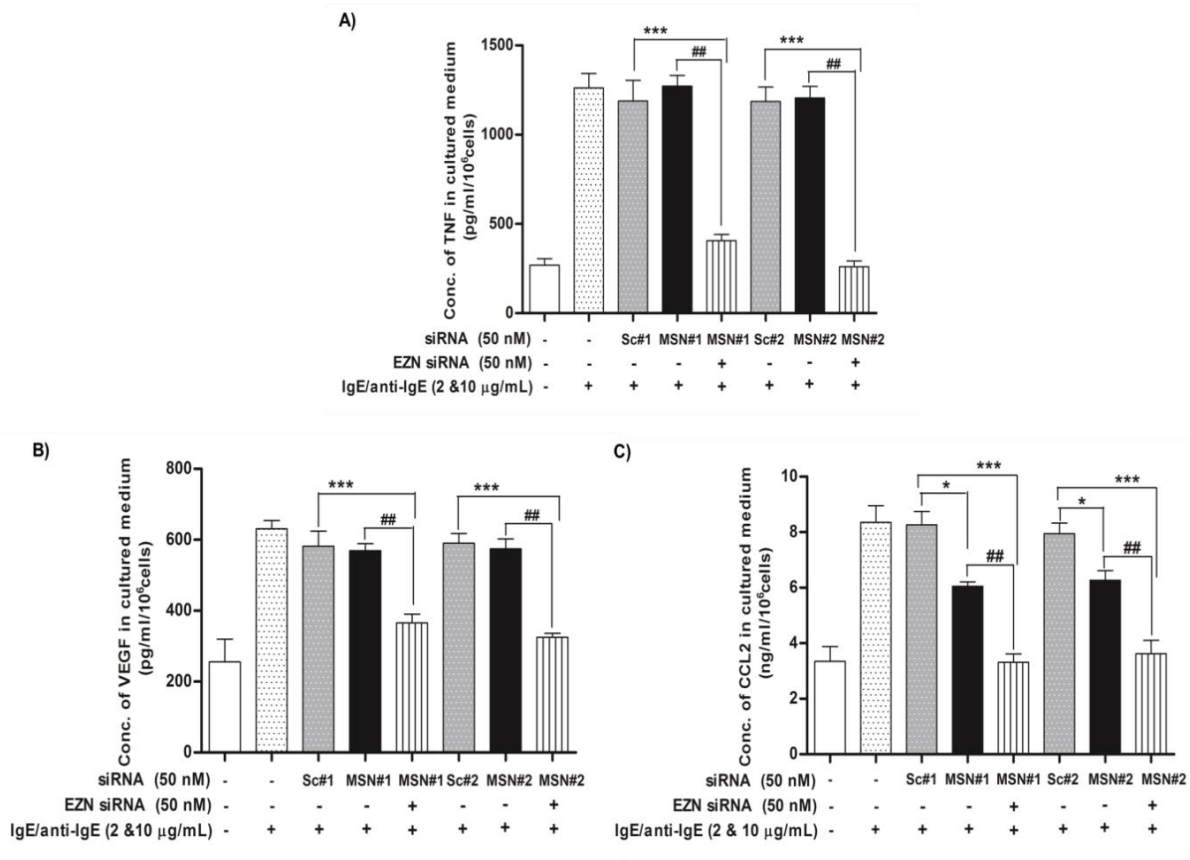


Figure 4.3. The effect of MSN & EZN siRNA induced knockdown on pro-inflammatory mediator release from human MC stimulated by IgE/anti-IgE. LAD2 MC transfected with siRNAs targeting MSN (#1 & #2) and/or EZN or the scramble siRNA (Sc) #1 & #2 controls as described in Fig. 1.1, ($0.1x10^6$ cells) were stimulated with IgE/anti-IgE (2&10 μg/mL) for 24 h to measure release of the cytokines (A) TNF and (B) VEGF, as well as the chemokine (C) CCL2. All conditions were performed in triplicates for each data set, repeated three times (n=3) and results presented as mean ± SD. Significance of comparisons is denoted by $p<0.001$ (##) for each pairwise comparison made between stimulated LAD2 MC transfected with MSN siRNA and MSN & EZN siRNAs, as well as by $p<0.0001$ (***) for each pairwise comparison made between stimulated scramble siRNA (Sc) controls and stimulated LAD2 MC transfected with MSN siRNA.

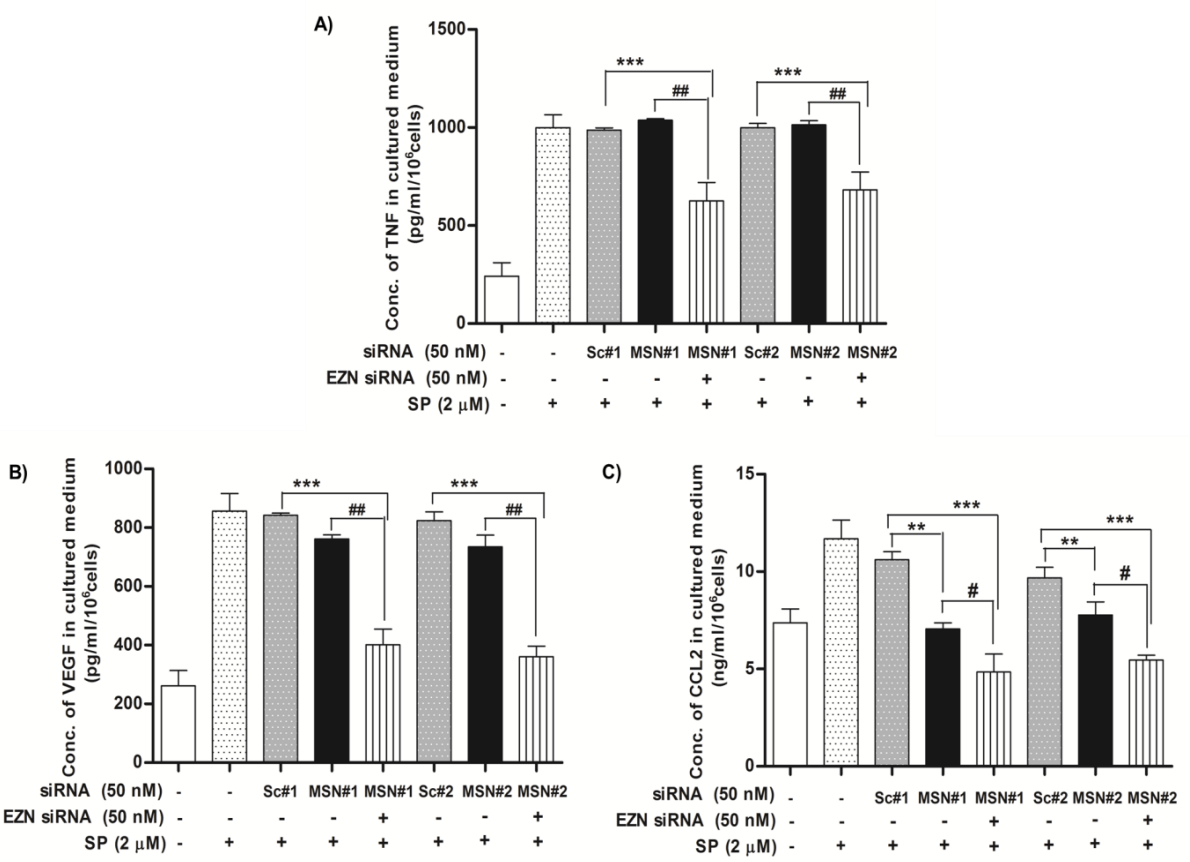


Figure 4.4. The effect of MSN & EZN siRNA induced knockdown on pro-inflammatory mediator release from human MC stimulated by SP. LAD2 MC transfected with siRNAs targeting MSN (#1 & #2) and/or EZN or the scramble siRNA (Sc) #1 & #2 controls as described in Fig. 1.1, ($0.1x10^6$ cells) were stimulated with SP (2 μM) for 24 h to measure release of the cytokines (A) TNF and (B) VEGF, as well as the chemokine (C) CCL2. All conditions were performed in triplicates for each data set, repeated three times (n=3) and results presented as mean ± SD. Significance of comparisons is denoted by $p<0.001$ (##) for each pairwise comparison made between stimulated LAD2 MC transfected with MSN siRNA and MSN & EZN siRNAs, as well as by $p<0.0001$ (***) for each pairwise comparison made between stimulated scramble siRNA (Sc) controls and stimulated LAD2 MC transfected with MSN siRNA.

Activation of moesin in human MC after neuropeptide stimulation is decreased by PI3K/mTOR inhibitors, and the flavonoids luteolin and methoxyluteolin

To investigate whether moesin is activated in human LAD2 MC in response to stimulation by the peptide SP, the phosphorylation (p) levels of moesin (pmoesinThr558), a signal associated with activated moesin conformation and total moesin were assessed by Western blot.

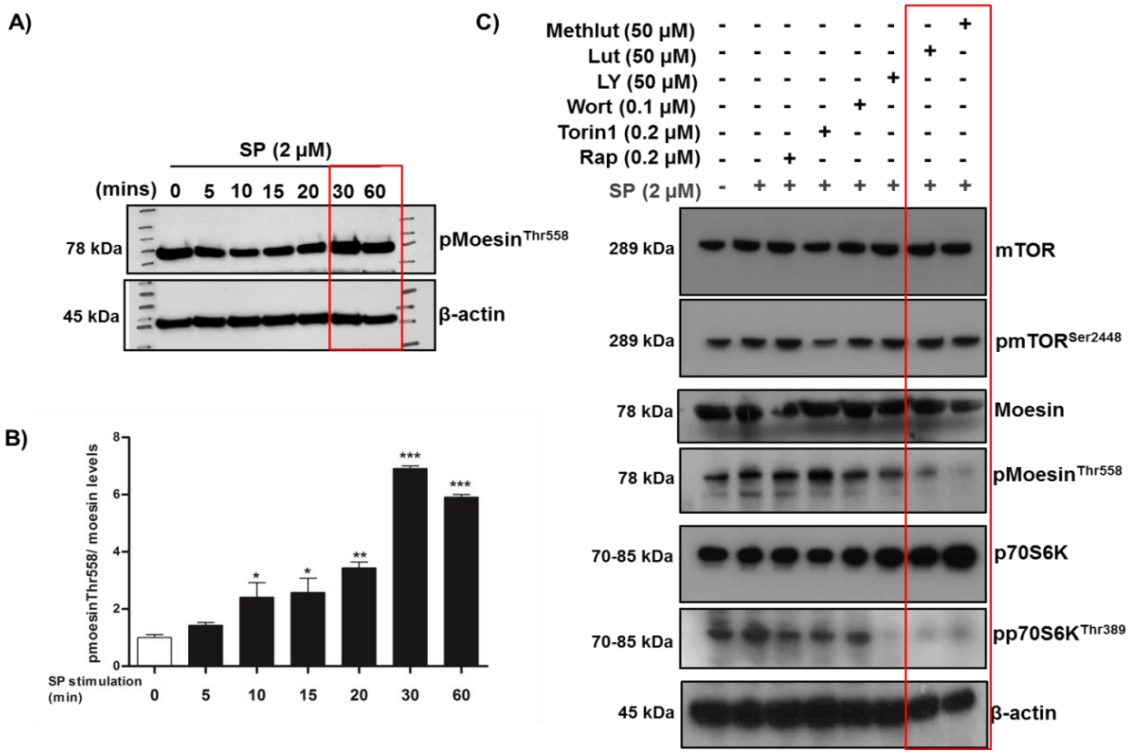


Figure 4.5. Activation of moesin in LAD2 MC stimulated with neuropeptides and the effect of pharmacological inhibition of PI3K/mTOR and flavonoids. LAD2 MC ($1x10^6$ cells) were stimulated with (A) SP (2 μM) for 0-60 mins to probe for the total and phosphorylated levels of moesin by Western blot analysis. (B) The ratios of pmoesinThr558 to total moesin are shown using densitometric analysis. C) LAD2 MC ($1 x10^6$ cells) were pre-treated with the mTOR inhibitors (rapamycin, Rap and Torin1, 0.2 μM, 24 h) or the upstream PI3K inhibitors (Wortmannin, Wort, 0.1 μM, 24h and LY294002, LY, 50 μM, 2 h) or the natural flavonoids (luteolin, Lut and methoxyluteolin, Methlut, 50 μM, 2 h), prior to stimulation with SP (2 μM). The total protein and phosphorylated levels of mTOR and substrate p70S6K, as well as moesin were analyzed by Western blot. β-actin served as the loading control.

Stimulation of LAD2 MC by SP (2 µM) for 0-60 min increased significantly increased levels of pmoesinThr558 after 10 min, which peak after 30 min of SP -stimulation (Figure 4.5 A&B).

To further evaluate the involvement of mTOR for moesin activation, denoted by pmoesinThr558 in LAD2 MC stimulated with SP, pharmacological inhibition of PI3K/mTOR signaling was done using specific inhibitors. LAD2 MC were pre-treated with the mTORC1 inhibitor rapamycin (0.2 µM, 24 h), the potent dual mTORC1/mTORC2 inhibitor Torin1 (0.2 µM, 24 h), the upstream PI3K inhibitors Wortmannin (0.1 µM, 24 h and LY294002 (50 µM, 2 h), prior to stimulation with SP for 30 mins. Western blot analysis revealed that pre-treatment with all inhibitors decreases the levels of pmTORSer2448 and the downstream mTORC1 substrate, pp70S6KThr389, when compared to SP-stimulated LAD2 MC (Figure 5.5 C).

Pretreatment of LAD2 MC with the flavonoids luteolin and methoxyluteolin (50 µM, 2 h), prior to stimulation with SP (2 µM) for 20 mins also decreases the levels of levels of pmTORSer2448 and the downstream mTORC1 substrate, pp70S6KThr389, as well as pmoesinThr558 (Figure 5.5 C), indicating the involvement of mTOR signaling in the activation of moesin.

4.4. Discussion

Here, we report that the ezrin protein of the ERM is primarily involved in the regulation of MC degranulation and for *de novo* synthesized release of TNF, VEGF and CCL2 from human MC in response to either the allergic (IgE/anti-IgE) or the peptide (SP) stimulation. We also report a novel finding that the pharmacological inhibition of the PI3K/mTOR signaling, and the flavonoids luteolin and methoxyluteolin decrease pmoesinThr588 levels that is critical for moesin activation, suggesting that mTOR signaling and moesin are also key regulators of MC mediator release. Our findings indicate that ezrin and moesin mediates F-actin rearrangement and cytoskeletal control for MC granular or vesicular release and are in agreement with the recent report showing that FcεRI-mediated allergic degranulation and histamine release is decreased in MC derived from ezrin-deficient mice (Staser, et al., 2013).

Previous studies have shown that F-actin polymerization is critical in setting the threshold for MC signaling via aggregation of FcεRI allergic receptors (Tolarova, et al., 2004). Ezrin and/or moesin could serve the function of bridging the clustered high affinity IgE receptor FcεRI in human MC (Rivera, et al., 2008;Rivera and Olivera, 2008;Modena, et al., 2016) to the cytoskeleton, in a similar manner to that reported for focal adhesion proteins and IgE-FcεRI for the activation of rat basophilic MC (Torres, et al., 2008). In addition, moesin and other ERM could also be implicated in the control of clathrin-mediated endocytosis (Barroso-Gonzalez, et al., 2009;Nomachi, et al., 2013), an important pathway for the internalization and recycling of GPCRs, such as those for the SP neurokinin receptor family in human MC (Kulka, et al., 2007) to regulate peptide-induced signaling.

Furthermore, surface expressed ezrin and moesin on human MC could also associate with FcεRI or peptide receptors bound to ligands, such as anti-IgE or SP in order to augment release of *de novo* synthesized TNF, VEGF and CCL2. In fact, the association of cell surface moesin and the toll-like receptor 4 (TLR4) for LPS is required for the LPS-stimulated TNF release from murine monocytes/macrophages (Iontcheva, et al., 2004;Zawawi, et al., 2010). Ezrin has also been identified as a crucial regulator for IL-10 release from B cells in response to LPS stimulation (Pore, et al., 2016) via a similar mechanism involving ligation with TLR4.

An important new finding here is that the flavonoids luteolin and methoxyluteolin, which inhibit activation of human MC, also decrease levels of pmTORSer2448, a site in mTOR phosphorylated by upstream phosphatidylinositol-3-kinase (PI3K)/mTOR signaling and the mTORC1 substrate p70S6K Thr389 site, as well as levels of pmoesinThr558, indicating inhibition of both mTOR signaling and the presence of the in-active moesin conformation. It can be inferred that upstream mTOR signaling is required for the phosphorylation of moesin at the Thr558 site, associated with the activation of ERM for cytoskeletal rearrangement (Matsui, et al., 1998;Nakamura, et al., 1999;Fievet, et al., 2004;Ben-Aissa, et al., 2012).

We speculate that the Rho family of GTPases (RhoA/Rac/Cdc42) (Hirao, et al., 1996;Mackay, et al., 1997;Brown, et al., 1998;Haas, et al., 2007;Oh and Jacinto, 2011;Liu, et al., 2010;Gulhati, et al., 2011;Azouz, et al., 2012;Higashio, et al., 2016), downstream of mTORC2 could regulate degranulation and release of TNF, VEGF and CCL2 from stimulated human MC. Dephosphorylation of the C-terminal site on moesin or down-regulation of the both ezrin and moesin proteins in human MC could decouple

the plasma membrane from the F-actin cytoskeleton and impair MC degranulation (Staser, et al., 2013) and vesicular mediator release. Noteworthy, the ERM exhibit functional redundancy in several cell types (Bretscher, et al., 2002;Fehon, et al., 2010), which could be explain our findings that down-regulation of moesin protein alone in MC did not have any significant decrease in both MC degranulation and *de novo* synthesized mediator release in response to IgE/anti-IgE or SP stimulation.

Moreover, stimulation of MC requires the rapid elevation of cytoplasmic calcium, and ezrin is essential for the regulation of calcium homeostasis *in vivo* (Hatano, et al., 2013). We had shown that luteolin and methoxyluteolin inhibit intracellular calcium levels in human MC (Weng, et al., 2015). Intracellular calcium initiates activation of PI3K and phospholipase C γ1 and γ2 (Gilfillan and Rivera, 2009) to activate PKC, contributing to regulation of granule exocytosis (Holowka, et al., 2012) and MC degranulation. Hence, it is likely that mTORC2 signaling involves AKT/mTORC1 activation and the ability to modulate phosphorylation of the PKC θ (Pietromonaco, et al., 1998;Sarbassov, et al., 2004) and Rho (Haas, et al., 2007;Jeon, et al., 2010;Saci, et al., 2011;Gulhati, et al., 2011) that are known to activate ERM (Hall, 2012) and reorganize the cytoskeleton for MC mediator release.

Previously, our laboratory had identified the inhibitory phosphorylation signals induced by flavonoids at several sites on moesin (Ser56/74 and/or Thr66) within the FERM domain of moesin (Kempuraj, et al., 2005), which were associated with inhibition of MC. It is possible that luteolin and methoxyluteolin could induce phosphorylation at these inhibitory sites in human MC, and also activate phosphatases to dephosphorylate the C-terminal Thr sites on ezrin and/or moesin to inhibit MC activation. Flavonoids were

also recently shown to inhibit the formation of SNARE complexes involved in MC degranulation, which include the Syntaxin 4 (Syn4)/ Synaptosome-associated protein 23 (SNAP-23) /Vesicle-associated membrane protein (VAMP) 2 and Syn4/SNAP-23/VAMP8 (Yang, et al., 2013).

The findings reported here provide an understanding of regulation of human MC activation by ezrin and moesin and further give mechanistic insights for the inhibition of MC degranulation and pro-inflammatory mediator release in the presence of luteolin and methoxyluteolin.

Chapter 5

TNF stimulation of IL-6, IL-8 and VEGF release from human keratinocytes involves mTOR activation, inhibited by methoxyluteolin

5.1. Introduction

Psoriasis is a chronic, inflammatory and autoimmune skin condition, characterized by increased keratinocyte proliferation and a disease pathology that involves the presence of skin lesions, plaques, redness and itching (Ozdamar, et al., 1996;Harvima, et al., 2008;Nestle, et al., 2009;Eberle, et al., 2016). The prevalence of psoriasis in the US is ~3 % (Helmick, et al., 2014), and is considered a major public health problem due to ineffective long-term treatments that resulted in the annual economic burden amounting to $11 billion in 2013 (Vanderpuye-Org, et al., 2015;Brezinski, et al., 2015). Recent studies on the pathogenesis of psoriasis have not only revealed a genetic component (Tsoi, et al., 2012), but also immune dysfunction (Li, et al., 2016b), as a consequence of an imbalance in polarized T-helper subsets (T_h1-T_h2-T_h17)(Nestle, et al., 2009;Eberle, et al., 2016) and activation of keratinocytes (Albanesi, et al., 2007;Harvima, et al., 2008;Eberle, et al., 2016), skin-resident and infiltrating MC (Toruniowa and Jablonska, 1988;Paus, et al., 2006;Harvima, et al., 2008;Metz and Maurer, 2009;Theoharides, et al., 2010) and other immune cells, including dendritic cells and macrophages (Deng, et al., 2016).

Thus, the increased levels of the pro-inflammatory cytokines tumor necrosis factor (TNF) (Olivieri, et al., 2009;Sedger and McDermott, 2014;Grine, et al., 2015), interferon gamma (IFNγ), interleukin (IL)-1β (Nickoloff, et al., 2007), IL-6(Hunter and Jones, 2015), IL-17 (Kirkham, et al., 2014), IL-22, IL-23 (Coimbra, et al., 2012;Raychaudhuri, et al., 1998;Suzuki, et al., 2014) and IL-33 (Theoharides, et al., 2010;Balato, et al., 2012), as well as angiogenic mediators, including vascular endothelial growth factor (VEGF) (Marina, et al., 2015)are all implicated in psoriasis. In addition, the

increased levels of neuropeptides, especially corticotropin releasing hormone (CRH) (Vasiadi, et al., 2012), neurotensin (NT) (Vasiadi, et al., 2012a) and substance P (SP) (Remröd, et al., 2007), could serve as the stimulus for activation of keratinocytes (Dallos, et al., 2006;Shi, et al., 2013;Shi, et al., 2011) and MC (Singh, et al., 1999;Donelan, et al., 2006;Kulka, et al., 2007;Theoharides, et al., 2010). Hence, neuroimmunoendocrine interactions involving neuropeptides, keratinocytes and MC could be important for the pathophysiology of psoriasis (Scholzen, et al., 1998;Paus, et al., 2006;Theoharides, et al., 2004b;Theoharides, et al., 2016b).

Considerable evidence has also shown that the phosphatidylinositol-3-kinase (PI3K)-dependent mammalian target of rapamycin (mTOR) kinase pathway is involved in the proliferation of epidermal keratinocytes (Mitra, et al., 2012) and activation in response to nerve growth factor (NGF) (Zhang and Ma, 2014) or 1L-17 and IL-22 (Datta, et al., 2013;Raychaudhuri and Raychaudhuri, 2014). The allergic stimulation and proliferation of MC is also mediated by PI3K/mTOR activation (Kim, et al., 2008a;Smrz, et al., 2011). Importantly, the elevated expression of mTOR and its phosphorylated substrates, indicating activation of pathway has been reported in human psoriatic lesional skin, as compared to non-lesional skin (Choi, et al., 1997;Buerger, et al., 2013;Chamcheu, et al., 2016).

However, the clinically available mTOR inhibitors, rapamycin (sirolimus) and its analog, everolimus, which have been tried for the treatment of psoriasis (Frigerio, et al., 2007;Wei and Lai, 2015) have shown low bioavailability/absorption profiles, and adverse side-effects, including skin irritability/rashes (Macdonald, et al., 2015), infections due to immunosuppresive properties of the drugs and acute renal failure (Ormerod, et al., 2005).

Other recent treatments for psoriais summarized in Table 1.2, include the anti-biologics for TNF (Kotsovilis and Andreakos, 2014;Cohen, et al., 2015), IL-17 (Ryoo, et al., 2016) and IL-23 (Gomez-Garcia, et al., 2016) are available, albeit the additional cost (Melnikova, 2009;Winterfield, et al., 2005), but are associated with the increased risk of infections and cardiovascular events (Winterfield, et al., 2005;Kaffenberger, et al., 2014;Deng, et al., 2016).

Hence, there is an urgent need for developing effective dual inhibitors for the abnormal activation of human MC and KC. Luteolin (3',4',5,7-tetrahydroxyflavone) not only inhibits MC-mediated allergic or neuropeptide pro-inflammatory responses (Asadi and Theoharides, 2012b;Kimata, et al., 2000a;Kritas, et al., 2013), but also MC-induced T cell activation (Theoharides, et al., 2007a;Kempuraj, et al., 2008). Our laboratory had shown that luteolin inhibits keratinocyte activation via mechanisms involving suppression of the nuclear factor kappa B (NF-κB) signaling and the subsequent gene expression and release of the pro-inflammatory mediators, IL-6, CXCL8 and VEGF (Weng, et al., 2014). More recently, we showed that its structural analog, 3',4',5,7-tetramethoxyflavone (methoxyluteolin) is a more potent MC inhibitor (Weng, et al., 2015), and is known to be metabolically more stable (Walle, 2007).

In the present study, we report a novel finding that mTOR activation is involved in the stimulation of human KC by TNF and that this mechanism is inhibited by the flavonoid methoxyluteolin, more potently than luteolin or the allosteric mTOR inhibitor rapamycin, after which the kinase was discovered.

5.2. Materials and Methods

Materials

Recombinant human TNF was purchased from R&D Systems (Minneapolis, MN) and dissolved in double-distilled water. The PI3K inhibitor LY294002 (Cell Signaling Technology, CST) and mTOR inhibitors rapamycin and PP242 (TOCRIS biosciences, Bristol, UK) were purchased. The flavonoids luteolin and methoxyluteolin were obtained from Pharmascience Nutrients (Clear Water, FL), dissolved in DMSO and used in cell culture at the final concentration of DMSO was < 0.1%; pH was 7.4. RNeasy Mini (Qiagen Inc., Valencia, CA) and iScript cDNA synthesis kits (BioRad, Hercules, CA) were purchased. Taqman gene expression primers/assays for IL-6 (Hs00985639_m1), CXCL8 (Hs00174103_m1), VEGF (Hs00900055_m1) and GAPDH endogenous control (4310884E) were purchased from Applied Biosystems (Foster City, CA). ELISA kits for TNF (DY210), CXCL8 (DY208) and VEGF (DY293B) were purchased from R&D Biosystems (Minneapolis, MN). Rabbit mAb for mTOR (7C10), pmTORSer2448 (D9C2), pmTORSer2481, mTORC1 substrates p70S6K (49D7), pp70SK Thr389 (108D2), 4EBP1 (53H11) and p4EBP1Thr37/46 (236B4), as well as the loading control β-actin (D6A8) were purchased from Cell Signaling Technology (Beverly, MA).

Methods

Human keratinocyte (KC) Culture

The immortalized human keratinocyte cell line HaCaT was used since it is suitable for studies relevant to Ps (Belso, et al., 2008;Zampetti, et al., 2009). HaCaT keratinocytes were kindly provided by Dr. A. Slominski (University of Tennessee, Memphis, TN) and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Sigma-Aldrich). Adult normal human epidermal keratinocytes (NHEKs) were purchased from Life Technologies (Carlsbad, CA) and cultured in EpiLife serum-free medium containing Human Keratinocyte Growth Supplement (HKGS, Life Technologies). Cells were maintained at 37 °C in 5% CO_2 incubator. Cell viability was determined by trypan blue (0.4%) exclusion test.

Human KC Treatments

HaCat KC were stimulated with TNF (10 or 50 ng/mL, R&D Systems) and/or pre-treated with the following inhibitors: (a) PI3K inhibitor [LY294002 (LY), 1-50 µM, 2 h, Cell Signaling Technology], (b) mTOR inhibitors [rapamycin (Rap), 0.005-0.2 µM or PP242 (PP) 0.005-0.2 µM TOCRIS biosciences, Bristol, UK] and (c) the flavonoids [luteolin (Lut) or methoxyluteolin (Methlut), 1-50 µM, 2,12 or 24 h, PharmaScience Nutrients, Clearwater, FL]. All inhibitors were dissolved in water or DMSO with final volume of < 0.1 % added to cells, while control cells were pre-treated with 0.1% DMSO only.

Pro-inflammatory Mediator Release

Mediator release in cell-conditioned culture medium/ supernatant fluid was determined by using commercial available ELISA kits (R&D Systems, Minneapolis,

MN) as per manufacturer's instructions. Human KC (5×10^4 cells per well) were seeded in 12-well plates (Becton Dickinson, Franklin Lakes, NJ) and allowed to grow overnight prior to pre-treatment with inhibitors/flavonoids (for 30 mins) and/or stimulation with TNF (50 ng/mL, 24 h). KC supernatant fluids were collected to measure IL-6, CXCL8 and VEGF mediator release. For all experiments, the control cells were treated with equal volume of culture medium or DMSO. The minimum detectable level for all mediators by ELISA was 5 pg/mL.

Pro-inflammatory Mediator Gene Expression

Gene expression analysis was carried out by quantitative real-time PCR (qRT-PCR) using Taqman assays (Applied Biosystems, Carlsbad, CA) to assess the expression of IL-6, IL-8 and VEGF mediators. HaCat KC (2.5×10^5 cells) were seeded in 6-well plates (Becton Dickinson, Franklin Lakes, NJ) and allowed to grow overnight prior to pre-treatment with inhibitors/flavonoids (for 30 mins) and/or stimulation with TNF (50 ng/mL, 6 h). Total RNA from HaCat KC was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription (RT) was performed with 300 ng of total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Taqman gene expression assays were performed using validated oligonucleotide primers (Applied Biosystems). Samples were run at 45 cycles using Applied Biosystems 7300 Real-Time PCR System. Relative mRNA abundance was determined from standard curves run for each experiment. Gene expression was normalized to GAPDH as the endogenous control.

Assessing mTOR Activation by Western blot & ELISA

The activation of mTOR was assessed by phosphorylation (p) of mTOR (pmTORSer2448) and the downstream mTORC1 substrates, p70S6K (pp70S6KThr389) and 4EBP1 (p4EBP1Thr37/46) using both Western blot analysis and Cell Signaling Technology (CST) PathScan Phospho-ELISA kits (CST, Danvers, MA). HaCat KC (2×10^6/condition) were seeded in 100 mm dishes (Sigma Aldrich) for 24 h, serum-starved overnight (or then pre-treated with inhibitors and flavonoids), then stimulated with TNF (50 ng/mL) for 0-60 min, before cell lysates were collected. The total protein concentrations were determined by the bicinchoninic acid assay (Thermo Fisher Sci.) using bovine serum albumin protein (BSA) as standards. The total cellular proteins (20 or 40 µg) were separated using 4-20 % Mini-PROTEAN TGX™ precast gels (BioRad, Hercules, CA) under SDS denaturing conditions and electro-transferred onto PVDF membranes (Millipore, Carrigtwohill, Ireland). Blocking was carried out with 5% (w/v) BSA in Tris-buffered saline (TBS) containing 0.1 % Tween-20. The membranes were probed with the following primary antibodies: mTOR (7C10), phospho-mTOR (Ser2448), phospho-mTOR (Ser2481), mTORC1 substrates p70S6K, phospho-p70S6K (Thr389; Ser371), 4EBP1 and phospho-4EBP1 (Thr-37/46), as well as β-actin, which served as the loading control (Cell Signaling Technology). All proteins were visualized with horseradish peroxidase-conjugated secondary antibodies and then by enhanced chemiluminescence (SuperSignal West Pico, Thermo Fisher Sci). To quantitate changes in the levels of phosphorylated proteins after Western blot analysis, densitometric analysis was done using Image J software to present ratios of 1) pmTORSer2448 to total mTOR, 2) pp70S6K Thr389 to total p70S6K and 3) p4EBP1to total 4EBP1.

Statistical Analysis

All conditions were performed in triplicate and all experiments were repeated at least three times (n=3). Results from cultured cells are presented as mean ± SD. Comparisons were made between (1) control and stimulated cells using the unpaired 2-tailed, Student's *t*-test, with significance of comparisons denoted by the horizontal lines and by $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***). Multiple comparisons were also made between (1) for all conditions with stimulated cells and with inhibitors using one-way ANOVA, followed by post-hoc analysis by Dunnett's Multiple Comparison Test; significance is denoted by horizontal lines and indicated by $p<0.001$ or $p<0.0001$ and (2) all the inhibitors/flavonoids among themselves using one-way ANOVA, followed by post-hoc analysis by Tukey's Multiple Comparison Test; those conditions for which there is significance denoted by the horizontal brackets and by the corresponding $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***) values. These analyses were performed using the GraphPad Prism version 5.0 software (GraphPad Software, San Diego, CA, USA). Representative images for western blots were scanned and analyzed using Image J (NIH, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2015).

5.3. Results

TNF-stimulated human KC pro-inflammatory mediator release is inhibited by PI3K or mTOR inhibitors

Initially, we investigated the optimal concentrations of TNF (1-100 ng/mL) that stimulated the pro-inflammatory mediator release from cultured human HaCat KC and NHEK by using enzyme-linked immunosorbent assays (ELISAs). Stimulation by TNF at dose of 10 or 50 ng/mL significantly increased the release of IL-6, CXCL8 and VEGF from human KC after 24 h (data not shown). We next evaluated the involvement of mTOR signaling by using pharmacological inhibitors of mTOR, which included the bacterial macrolide and more selective mTORC1 inhibitor rapamycin and the potent ATP-competitive inhibitor of both mTOR complexes, PP242, as well as the upstream PI3K inhibitor LY294002, prior to stimulation with TNF. Pre-treatment of KC with inhibitors of mTOR signaling, rapamycin and PP242 (0.01-1 μM) for 2, 6, 12 or 24 h had no effect on TNF-stimulated mediator release. Inhibitory dose-dependent and time course studies revealed that rapamycin (5 μM, 24 h) and PP242 (2.5 μM, 1 h) significantly decrease ($p<0.001$) TNF-stimulated IL-6, CXCL8 and VEGF release from HaCat KC (Fig 5.1). Pre-treatment with the PI3K inhibitor, LY294002 (50 μM, 1 h), following TNF-stimulation of HaCat KC, also decreases ($p<0.001$) TNF and CXCL8 release.

TNF-stimulated human KC mediator release is inhibited by the flavonoids luteolin and methoxyluteolin

We further compared the effects of the PI3K or mTOR inhibitors and the flavonoids luteolin and methoxyluteolin on HaCat KC mediator release after TNF stimulation for 24 h. HaCat KC were pre-treated with the natural flavonoids luteolin or

methoxyluteolin (50 µM, 1 h), then stimulated by TNF (50 ng/mL) for 24 h to measure inhibitory effect on mediator release by ELISA. The release of TNF, CXCL8 and VEGF significantly decreases in the presence of the flavonoids ($p<0.0001$), after TNF-stimulation, and inhibits pro-inflammatory mediator release better than rapamycin or LY294002 (Fig. 5.1).

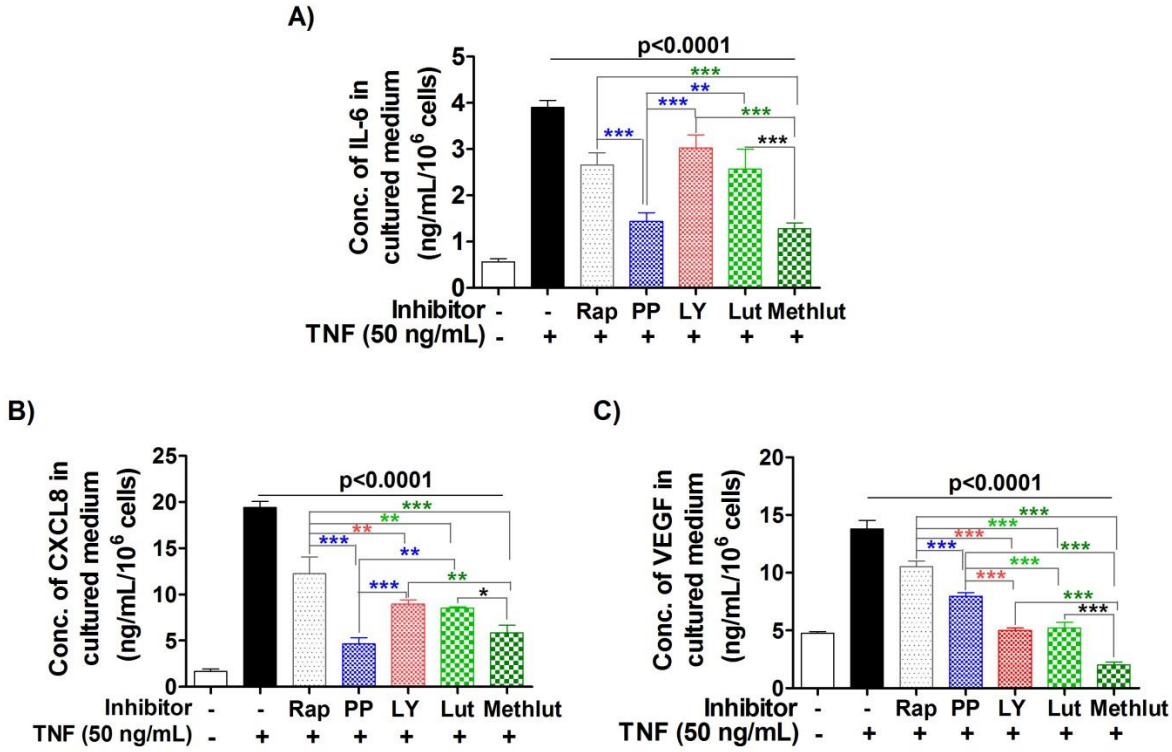


Figure 5.1. Human KC pro-inflammatory mediator release in response to TNF is attenuated by PI3K/mTOR inhibitors and luteolin or methoxyluteolin. HaCat KC (1 x10^5 cells) were pre-treated with the mTOR inhibitors [rapamycin (Rap) 5 µM, 24 h and PP242 (PP) 2.5 µM, 2 h] or the upstream PI3K inhibitor [LY294002 (LY) 50 µM, 2 h] or the natural flavonoids [luteolin (Lut) and methoxyluteolin (Methlut) 50 µM, 30 mins], then stimulated with TNF (50 ng/mL) for 24 h to measure release of (A) TNF, (B) CXCL8 and (C) VEGF mediators by specific enzyme-linked immunosorbent assays (ELISA). All conditions were performed in triplicates for each data set and were repeated three times (n=3). Significance of comparisons were made for stimulated cells and those with inhibitors/flavonoids, as denoted by the horizontal lines ($p<0.0001$) and also among each of the inhibitors/flavonoids treatments shown by the horizontal brackets and by corresponding $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).

HaCat KC were also pre-treated with luteolin and methoxyluteolin at various doses (10 or 50 µM) for 0.5, 1, 6 h, then stimulated by TNF (50 ng/mL) for 24 h to measure release of pro-inflammatory mediators.

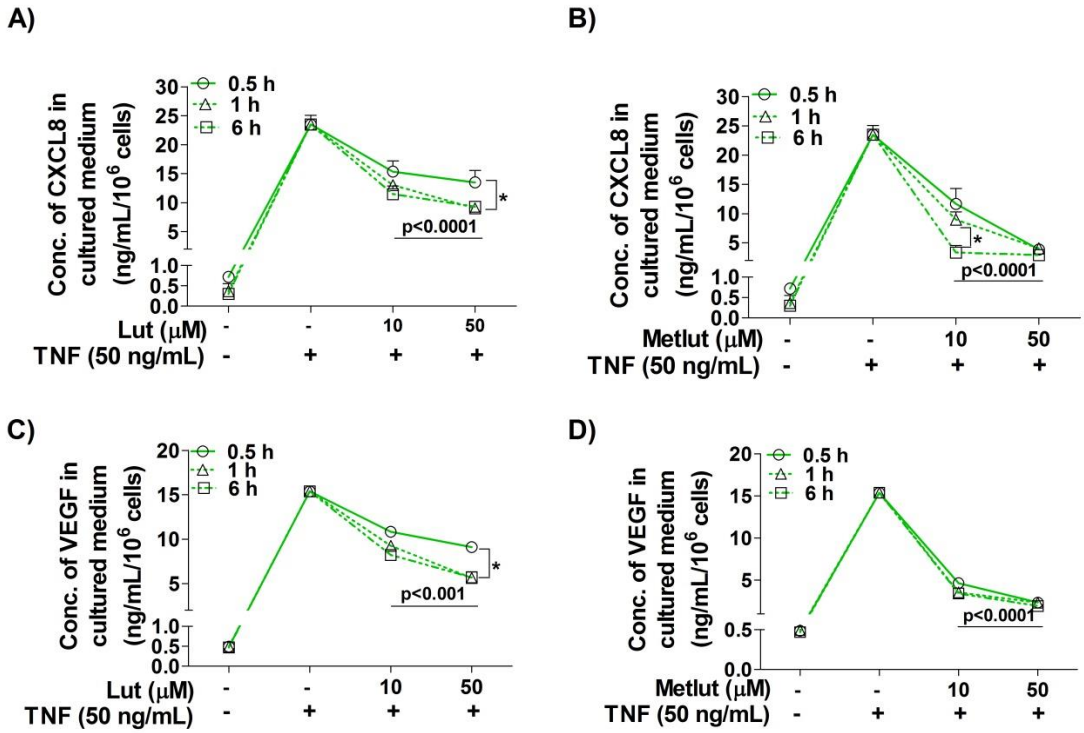


Figure 5.2. Methoxyluteolin more potently than luteolin inhibits TNF-stimulated pro-inflammatory CXCL8 and VEGF release from human KC. LAD2 MC (0.5 x10^6 cells) were pre-treated the flavonoids A) and B) luteolin (Lut) 1-50 µM and methoxyluteolin (Methlut), 1-50 µM] for 30 min, then stimulated with NT (10 µM) for 24 h to measure release of **(A)** TNF, **(B)** CXCL8 and **(C)** VEGF by ELISA. All inhibitors were dissolved in water or DMSO with final concentration < 0.1 %. All conditions were performed in triplicates for each data set and were repeated three times (n=3). Significance of comparisons were made for stimulated cells and those with flavonoids, as denoted by the horizontal lines ($p<0.001$ or $p<0.0001$) and also among each of the flavonoids treatments shown by the vertical brackets and by corresponding $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).

The release of CXCL8 and VEGF significantly decreases ($p<0.001$) in the presence of the flavonoids at 10 or 50 µM, while methoxyluteolin is a more potent

inhibitor than luteolin ($p<0.001$) at equimolar flavonoid concentrations from TNF-stimulated HaCat KC, with maximal flavonoid inhibition at 50 µM (Fig. 5.2).

We also investigated the involvement of mTOR signaling in pro-inflammatory mediator release from NHEK in response to TNF by ELISAs. Stimulation by TNF at dose of 50 ng/mL significantly increased ($p<0.001$) the release of IL-6 and CXCL8, but not VEGF from NHEK after 24 h (Fig 5.3).

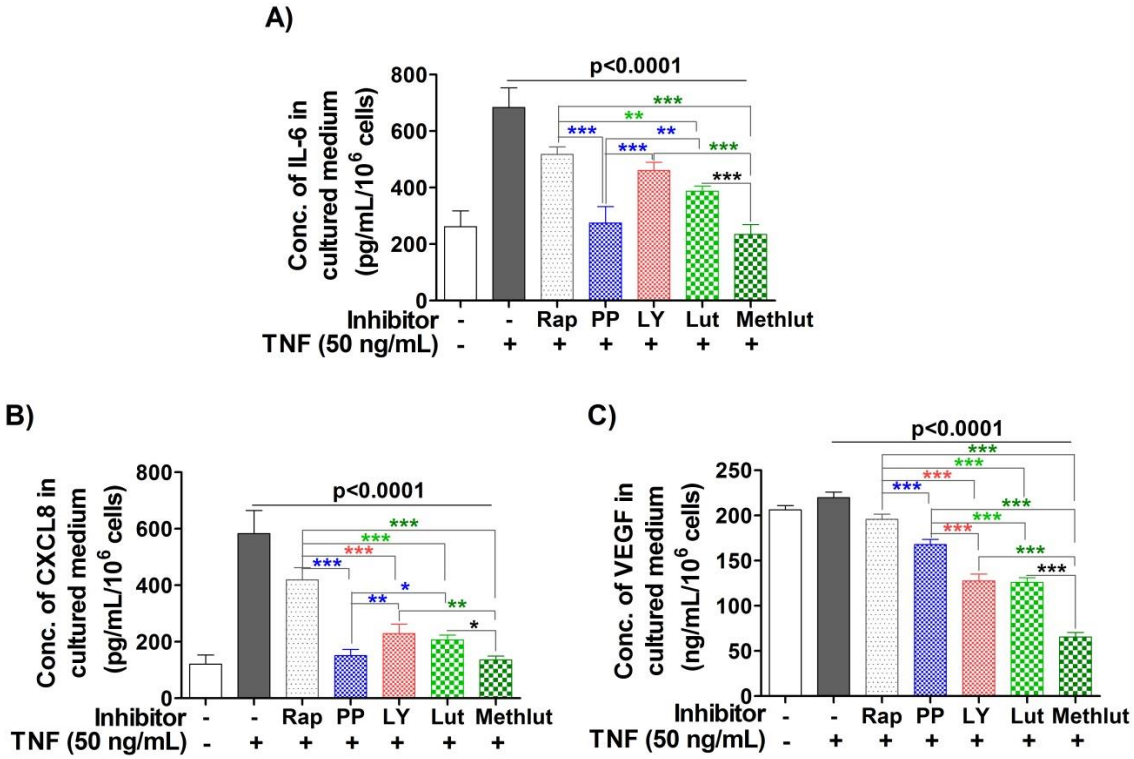


Figure 5.3. Human NHEK pro-inflammatory mediator release in response to TNF is attenuated by PI3K or mTOR inhibitors and luteolin or methoxyluteolin. NHEK (1 x10^5 cells) were pre-treated with the mTOR inhibitors (rapamycin, Rap 0.1 µM, 24 h and PP242, PP, 0.05 µM, 2 h) or the upstream PI3K inhibitor (LY294002, 20 µM, 2 h) or the natural flavonoids (luteolin, Lut and methoxyluteolin, Methlut, 10 µM, 30 mins), then stimulated with TNF (50 ng/mL) for 24 h to measure release of **(A)** TNF, **(B)** CXCL8 and **(C)** VEGF mediators by specific enzyme-linked immunosorbent assays (ELISA). All conditions were performed in triplicates for each data set and were repeated three times (n=3). Significance of comparisons were made for stimulated cells and those with inhibitors/flavonoids, as denoted by the horizontal lines ($p<0.001$ or $p<0.0001$) and also

among each of the inhibitors/flavonoids treatments shown by the horizontal brackets and by corresponding $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).

Pre-treatment with the mTOR inhibitors (rapamycin, 0.1 µM, 24 h or PP242, 0.05 µM, 2 h) and the PI3K inhibitor, LY294002 (10 µM, 1 h), significantly decreases ($p<0.001$) IL-6 and CXCL8 release from NHEK stimulated by TNF. Pre-treatment with the flavonoids luteolin and methoxyluteolin (10 µM, 1 h) also decreases all pro-inflammatory mediator release, including the VEGF release from non-stimulated cells.

TNF-stimulated pro-inflammatory mediator gene expression in human KC is decreased by the PI3K/mTOR inhibitors, and the flavonoids luteolin and methoxyluteolin

We further investigated whether mTOR signaling is involved in the production of pro-inflammatory mediators in human KC after TNF stimulation by measuring mediator gene levels using quantitative real-time PCR (qRT-PCR). HaCat KC were pre-treated with the mTOR inhibitors rapamycin (5 µM, 24 h) and PP242 (2.5 µM, 1 h) and the PI3K inhibitor LY294002 (50 µM, 1 h), prior to stimulation with TNF (50 ng/mL) for 6 h. Stimulation of HaCat KC by TNF significantly increases ($p<0.0001$) the gene expression of TNF, CXCL8 and VEGF after 6 h, which all decrease ($p<0.0001$) after treatment with the PI3K or mTOR inhibitors (Fig. 5.4).

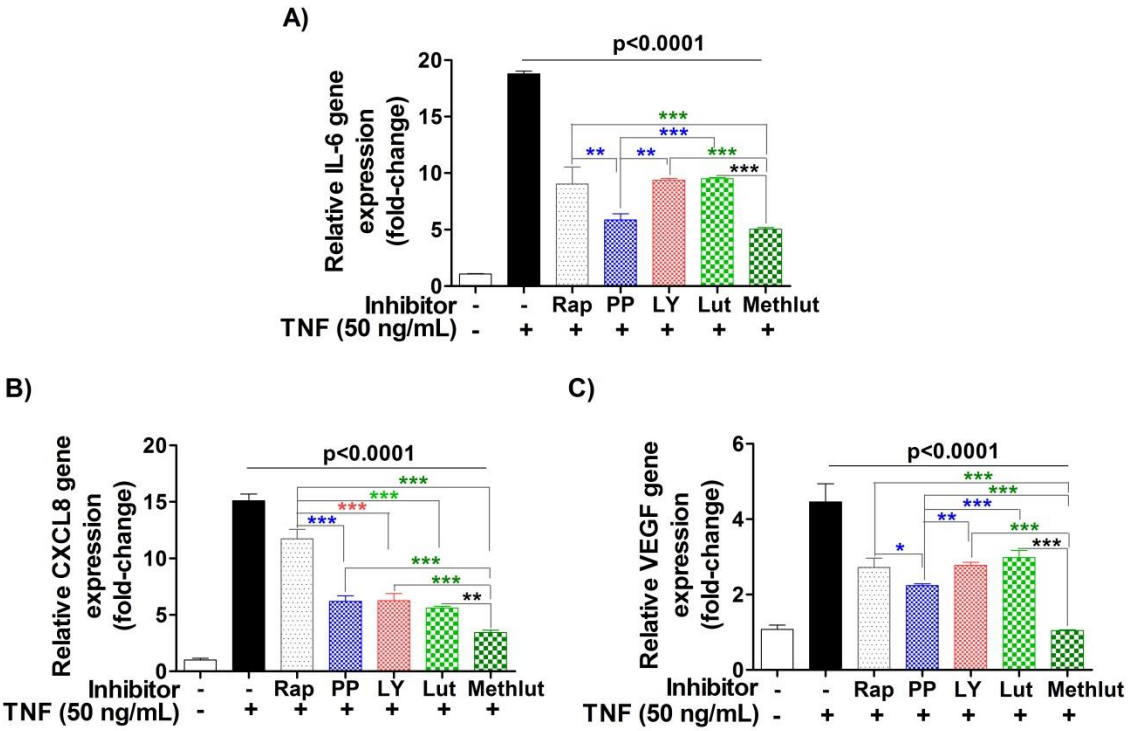


Figure 5.4. HaCat pro-inflammatory mediator gene expression in response to TNF is inhibited by the PI3K or mTOR inhibitors and luteolin and methoxyluteolin. HaCat KC ($0.2x10^6$ cells) were pre-treated with the mTOR inhibitors (rapamycin, Rap (5 µM, 24 h and PP242, PP, 2.5 µM, 1 h) or the PI3K inhibitor (LY294002, LY 50 µM, 1 h) or the natural flavonoids (luteolin, Lut and methoxyluteolin, Methlut, 50 µM, 1 h), prior to stimulation with TNF (50 ng/mL) for 6 h to measure gene expression of (A) TNF, (B) CXCL8 and (C) VEGF by RT-qPCR. All inhibitors were dissolved in water or DMSO with final concentration < 0.1 %. All conditions were performed in triplicates for each data set and were repeated three times (n=3). Significance of comparisons were made for stimulated cells and those with inhibitors/flavonoids, as denoted by the horizontal lines ($p<0.0001$) and also among each of the inhibitors/flavonoids treatments shown by the horizontal brackets and by corresponding $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).

To determine the inhibitory effect of the flavonoids luteolin and methoxyluteolin on pro-inflammatory gene expression in TNF-stimulated KC, and compare these to the effect of PI3K or mTOR inhibitors, HaCat KC were pre-treated with luteolin and methoxyluteolin (50 µM, 1 h), followed by stimulation with TNF (50 ng/mL) for 6 h. Pre-treatment of HaCat KC with luteolin and methoxyluteolin significantly decreases

($p<0.0001$) the gene levels TNF, CXCL8 and VEGF after 6 h in response to TNF (Fig. 5.4). Methoxyluteolin (50 µM) more potently ($p<0.0001$) than luteolin or PI3K and mTOR inhibitors decreases this pro-inflammatory gene expression in HaCat KC after TNF stimulation.

TNF stimulation of human KC activates signaling via mTORC1 that is inhibited by methoxyluteolin better than luteolin

To investigate whether mTOR signaling increases HaCat KC in response to NT to TNF stimulation, Western blot analysis were performed to detect the total and phosphorylated (p) levels of mTOR and its substrates p70S6K and 4EBP1 proteins (Fig. 5.5A). Stimulation of HaCat KC with TNF (50 ng/mL) increases the levels of pmTORSer2448 and the downstream mTORC1 substrates, pp70S6KThr389 and p4EBP1Thr37/46, which peak at 15 min, when compared to control cells.

We further evaluated the effects of luteolin and methoxyluteolin on mTOR signaling in human KC in response to TNF by comparing their inhibition to those treated with the PI3K and mTOR inhibitors. HaCat KC were serum-starved overnight, then pre-treated with rapamycin (5 µM, 24 h) or PP242 (2.5 µM, 1 h) or LY294002 (50 µM, 1 h) or luteolin and methoxyluteolin (50 µM, 1 h) prior to stimulation with TNF (50 ng/mL) for 15 min. Pre-treatment with PI3K or mTOR inhibitors and luteolin and methoxyluteolin, prior to stimulation with TNF significantly decreases levels of pmTORSer2448, pp70S6KThr389 and p4EBP1Thr36/47, when compared to those of TNF-stimulated HaCat KC.

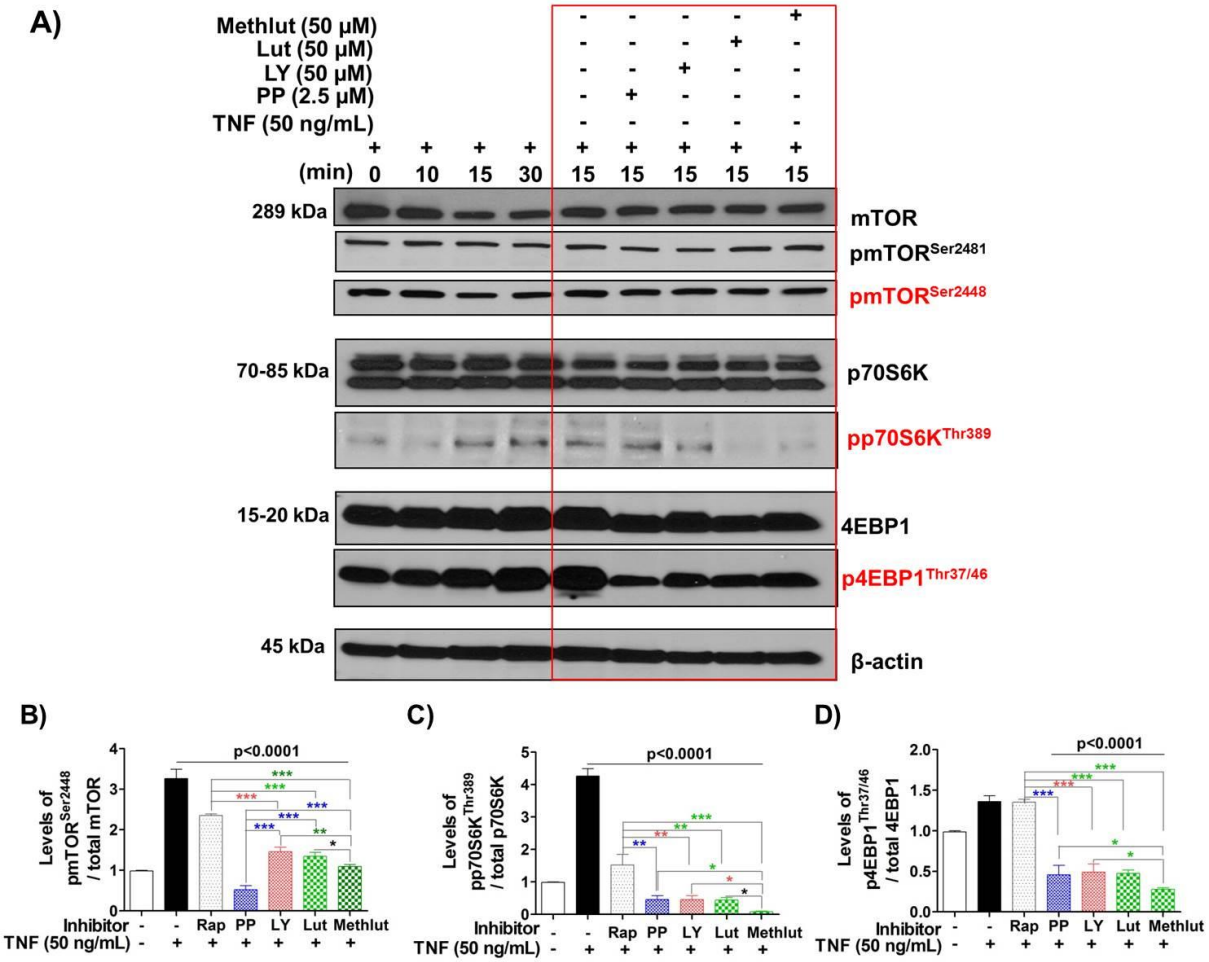


Figure 5.5. TNF-stimulated mTOR activation in HaCat KC is inhibitable by luteolin and methoxyluteolin. HaCat KC ($2x10^6$ cells) were serum-starved overnight, then stimulated with TNF (50 ng/mL) for 0-30 mins to probe for the total and phosphorylated levels of mTOR, and substrates p70S6K and 4EBP1 by Western blot analysis; **(A)** peak mTOR activation is denoted at 15 min from the increased levels of pmTORSer2448 and pp70S6KThr389 proteins, when compared to control cells. HaCat KC were also pre-treated with the inhibitors for mTOR (rapamycin, Rap, 5 µM, 24 h and PP242, PP, 2.5 µM) and for PI3K (LY294002, LY, 50 µM, 2 h), as well as the natural flavonoids (luteolin, Lut and methoxyluteolin, Methlut, 50 µM, 1 h), prior to stimulation with TNF (50 ng/mL) for 15 min. **(B)** The protein levels of mTOR, pmTOR Ser2448, p70S6K, pp70S6K Thr389, 4EBP1 and p4EBP1Thr37/46 were measured using specific total or phospho-ELISA kits for equal amounts of protein lysates, with ratios of phosphorylated to total proteins normalized. All conditions were performed in triplicates for each data set and were repeated three times (n=3). Significance of comparisons were made for stimulated cells and those with inhibitors/flavonoids, as denoted by the horizontal lines ($p<0.0001$) and also among each of the inhibitors/flavonoids treatments shown by the horizontal brackets and by corresponding $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).

To quantify the levels of phosphorylated mTOR and its substrates from the blots, densitometric analysis was performed on HaCat KC pretreated with the PI3K or mTOR inhibitors or the flavonoids luteolin and methoxyluteolin, prior to stimulation with TNF. Levels of pmTOR Ser2448 (Fig. 5.5B) and pp70S6K Thr389 proteins (Fig. 5C) in LAD2 MC pre-treated with inhibitors prior to NT or SP stimulation significantly decrease ($p<0.001$), as compared to NT or SP stimulation alone. Noteworthy, methoxyluteolin (50 µM) shows greater reduction of phosphorylated levels of pmTORSer 2448 and pp70S6KThr389 compared to luteolin (50 µM) or any of the other inhibitors.

5.4. Discussion

A novel finding reported here is the involvement of the PI3K/mTOR signaling cascade in the TNF stimulation of human keratinocytes, specifically in the NHEK and HaCat keratinocyte cell lines, resulting in the synthesis and release of pro-inflammatory IL-6, CXCL8 and VEGF mediators. Stimulation of human keratinocytes by TNF increased the levels of pmTORSer2448, the mTOR site phosphorylated by upstream PI3K/AKT signaling and the mTORC1-dependent p70S6KThr389 and p4EBP1Thr37/46 sites indicating activation of mTORC1 for protein translation (Laplante and Sabatini, 2012;Dibble and Cantley, 2015). Both the gene expression and release of IL-6, CXCL8 and VEGF in response to TNF stimulation of keratinocytes decreases in the presence of pharmacological inhibition of PI3K (LY294002) or mTOR (PP242>>rapamycin) signaling. Our findings are in agreement, with previous reports showing the involvement of PI3K/AKT/mTOR pathway in keratinocyte activation in response to NGF(Zhang and Ma, 2014) or 1L-17A and IL-22 (Datta, et al., 2013;Raychaudhuri and Raychaudhuri, 2014) stimulus to regulate expression of VEGF and angiogenesis.

An important new finding reported here is that the flavonoids luteolin and methoxyluteolin significantly inhibit *gene* expression and release of all pro-inflammatory mediators from TNF-stimulated human keratinocytes via inhibition of PI3K/mTORC1 pathway. Intriguingly, the structurally related flavonoid, fisetin has previously been shown to inhibit the PI3K/mTOR signaling in several cancer cell lines (Adhami, et al., 2012;Syed, et al., 2013). Previously, we had shown that luteolin inhibits keratinocyte activation by targeting NF-κB inactivation and the subsequent synthesis and release of the pro-inflammatory mediators, IL-6, CXCL8 and VEGF (Weng, et al., 2014). Our

present findings now add to these studies, by suggesting that mTOR is involved in the transcriptional regulation of mediator induction in human keratinocyte after TNF stimulation, possibly via NF-κB that is downstream of mTOR signaling (Dan, et al., 2008). Our findings are also in line with the previous studies showing that the suppression of AKT, mTOR and NF-κB pathways in TNF stimulated keratinocytes by another related flavonoid, myricetin decreases production of several pro-inflammatory mediators, including CXCL8 (Lee and Lee, 2016).

TNF is a well-known stimulus for the activation of NF-κB, which is implicated in the regulation of IL-6 (Vanden Berghe, et al., 2000), CXCL8 and VEGF gene synthesis in keratinocytes (Weng, et al., 2014), as well as reactive oxygen species (Young, et al., 2008). IL-17A has also been characterized as a pro-inflammatory stimulus for keratinocyte activation (Muromoto, et al., 2016), while a recent study further reported on synergistic action of TNF and IL-17A on NF-κB activation in human keratinocytes (Johansen, et al., 2016). Interestingly, the elevated expression of NF-κB subunits (Lizzul, et al., 2005;Weng, et al., 2014) and mTOR signaling components: pmTORSer2448 and downstream mTORC1 substrates, S6 kinase 1, pS6K1Ser235/6 (Choi, et al., 1997;Buerger, et al., 2013;Raychaudhuri and Raychaudhuri, 2014),4EBP1 and p4EBP1 (Kjellerup, et al., 2009) have been shown in psoriatic lesional skin, indicating activation of both mTOR and NF-κB signaling. Interestingly, psoriatic lesional skin is also positive for the expression of MC and its derived mediators (Toruniowa and Jablonska, 1988;Ackermann and Harvima, 1998;Jiang, et al., 2001), while the increased levels of FcεRI receptor, implicated in mediating MC allergic response has recently been reported (Yan, et al., 2016), indicating MC activation. Thus, MC, which uniquely store pre-formed

TNF could be the source of this cytokine that is involved in the pathogenesis of psoriasis (Olivieri, et al., 2009;Sedger and McDermott, 2014;Grine, et al., 2015).

Our findings further reveal that the pro-inflammatory mediator secretion from TNF stimulated cultured keratinocytes, could explain the upregulated expression of IL-6 (Grossman, et al., 1989), CXCL8 (Jiang, et al., 2001) and VEGF (Canavese, et al., 2010;Detmar, 2004) in psoriatic skin, where resident keratinocytes . TNF has been shown to stimulate gene expression and release of pro-inflammatory IL-1β from keratinocytes (Kutsch, et al., 1993), which could act in an autocrine/paracrine manner to stimulate IL-6 release from keratinocytes (Kupper, et al., 1989). In fact, neuropeptides elevated in psoriasis, including NT (Vasiadi, et al., 2012a) and SP (Remröd, et al., 2007) could potentially stimulate IL-1β release from keratinocytes (Ansel, et al., 1990;Shi, et al., 2011;Shi, et al., 2013;Moura, et al., 2014) and also stimulate MC activation (Donelan, et al., 2006;Kulka, et al., 2007;Theoharides, et al., 2010;Vasiadi, et al., 2012a). MC-derived TNF can further stimulate IL-33 release from keratinocytes (Balato, et al., 2012). Moreover, our studies has shown that IL-33 augments SP-stimulated VEGF release from human mast cells; IL-33 gene expression is also increased in lesional psoriatic skin (Theoharides, et al., 2010) and elevated serum IL-33 levels have also been reported in patients with psoriasis (Mitsui, et al., 2015). Hence, cross-talk between MC and keratinocytes, which can lead to the release of these pro-inflammatory mediators, may exacerbate processes involved in psoriasis.

The activation of the PI3K/AKT/mTOR pathway in keratinocytes in response to NGF(Zhang and Ma, 2014) or 1L-17A and IL-22 (Datta, et al., 2013;Raychaudhuri and Raychaudhuri, 2014) stimulus has been reported and shown to regulate expression of

VEGF involved in angiogenesis. MC-derived histamine can also increase NGF production from keratinocytes, while NGF, in turn, stimulates histamine release from MC, creating a positive feedback loop (Kanda and Watanabe, 2005). MC are also important for maturation of T_h17 cells, which are recognized as key cells in autoimmune disorders, such as psoriasis via secretion of IL-17A and IL-22 (O'Connor, Jr., et al., 2010). In addition, MC-derived TNF could induce T cell activation (Theoharides, et al., 2007a;Kempuraj, et al., 2008). Recent evidence further shows that MC release IL-17A (Suurmond, et al., 2011) and IL-33(Hsu, et al., 2010), which also stimulates keratinocyte activation. Thus, targeting inhibition of MC and keratinocyte activation, as well as their interaction could be effective therapy for psoriasis.

Interestingly, targeting PI3K/mTOR signaling has been an attractive therapeutic avenue (Raychaudhuri and Raychaudhuri, 2014;Huang, et al., 2014) for psorisis, since the dual inhibitor of PI3K/mTOR, NVP-BEZ235 has shown anti-mitotic effects on proliferation of both human keratinocytes (Raychaudhuri and Raychaudhuri, 2014) and MC (Blatt, et al., 2012). However, the clinically available mTOR inhibitors, sirolimus and everolimus block proliferation of keratinocytes, but do not effectively alter pro-inflammatory cytokine production (DeTemple, et al., 2016). We had previously reported that luteolin inhibits TNF stimulated release of pro-inflammatory mediators from keratinocytes, as well as their proliferation (Weng, et al., 2014). Luteolin, not only inhibits MC degranulation (Kimata, et al., 2000a) and cytokine release (Asadi and Theoharides, 2012), but also MC-induced T cell activation (Theoharides, et al., 2007a;Kempuraj, et al., 2008).

Our findings reveal that methoxyluteolin more potently than luteolin or the dual PI3K/mTOR inhibitors, inhibits the TNF stimulated keratinocyte responses. Moreover, we previously showed that methoxyluteolin can inhibit TNF synthesis and release in stimulated MC by inhibiting NF-κB activation (Weng, et al., 2015). Thus, methoxyluteolin provides a favorable treatment for inflammation of the skin found in psoriasis and other related skin disorders by targeting dual inhibition of aberrant MC and keratinocyte activation.

Chapter 6

Overall Conclusions and clinical significance

Overall Conclusions and clinical significance

6.1. NTR3/sortilin and mTOR: Potential Therapeutic Targets for Inflammation of the Brain in ASD, Inhibitable by Methoxyluteolin

The findings presented here extend on our previous reports of the elevated serum levels of the peptide neurotensin in children with ASD. We report that primary human microglia express only NTR3/sortilin and not the NTR1 or NTR2. NTR3/sortilin was previously shown to be expressed in the immortalized cell line of human (Martin, et al., 2003;Martin, et al., 2005) and murine (Dicou, et al., 2004) microglia. NT increases the gene expression and release of the IL-1β, CXCL8, CCL2 and CCL5 from human microglia via NTR3/sortilin. The increased levels of these pro-inflammatory cytokines and chemokines have been implicated in immune dysfunction associated with ASD (Vargas, et al., 2005;Ashwood, et al., 2011;Masi, et al., 2015;Choi, et al., 2016;Theoharides, et al., 2016a).

Stimulation of human microglia with NT also increases the gene expression of NTR3/sortilin and causes the receptor to be translocated from the cytoplasm to the cell surface, some of which is secreted extracellularly. Importantly, our findings show significantly elevated levels of NTR3/sortilin in the serum from children with ASD, compared to healthy controls. The source of the increased levels of serum NT in children with ASD is not yet known. The highest levels of serum NT we had measured before were present in those ASD children with reported gastrointestinal symptoms (Tsilioni, et al., 2014). On possibility is that serum NT derives from the gut, where it is known to be present (Dobner, et al., 1987;Carraway, et al., 1994) and increases permeability of the intestinal lumen (Castagliuolo, et al., 1999). NT may enter the blood and reach the brain

by stimulating perivascular MC (Miller, et al., 1995;Donelan, et al., 2006), implicated in the disruption of both the gut-blood barrier (Wallon, et al., 2008;Theoharides and Doyle, 2008) and the blood-brain barrier (BBB) (Theoharides, 1990;Esposito, et al., 2002;Ribatti, 2015) (Fig. 6.1).

Microglia-derived IL-1β, CXCL8, CCL2 and CCL5 in response to NT can then augment the activation of perivascular MC further disrupting the BBB (Theoharides, et al., 2004c;Takeshita and Ransohoff, 2012;Rochfort and Cummins, 2015), perhaps initiating a feed-back mechanism. Microglia are stimulated by MC-derived histamine (Dong, et al., 2014a) and tryptase (Zhang, et al., 2012b). Hence, communication between MC and microglia (Skaper, et al., 2012;Skaper, et al., 2014a), found to be activated in brains of patients with ASD (Vargas, et al., 2005;Morgan, et al., 2010;Rodriguez and Kern, 2011;Morgan, et al., 2014;Gupta, et al., 2014), is implicated in inflammation of the brain (Theoharides T.C., et al., 2016a). We also report that proliferation of human microglia-SV40 is increased by NT, which could be the stimulus for the increased microglial numbers found in brains of deceased ASD patients (Vargas, et al., 2005; Morgan, et al., 2010;Rodriguez and Kern, 2011;Morgan, et al., 2014).

NT-stimulation of human microglia-SV40 causes activation of the phosphatidylinositol-3-kinase (PI3K)-dependent mammalian target of rapamycin (mTOR) signaling kinase as shown by phosphorylation of mTORC1 substrates and inhibition of these responses by drugs that prevent mTOR activation. Importantly, the naturally occurring flavonoids 5, 7, 3', 4'-tetrahydroxyflavone (luteolin) and 3',4',5,7-tetramethoxyluteolin (methoxyluteolin) significantly inhibit either NT- or LPS-stimulated gene expression of all the pro-inflammatory mediators, as well as the activation of

mTORC1 and mTORC2 in human microglia. This suggests that mTOR signaling is involved in the transcriptional regulation of pro-inflammatory cytokine and chemokine synthesis in human microglia (Dan, et al., 2008;Saleiro and Platanias, 2015). Luteolin had been shown to inhibit pro-inflammatory responses in LPS-stimulated murine microglia by suppressing multiple pathways, including activation of mitogen-activated protein kinase and AKT (Zhu, et al., 2014), signal transducer and activator of transcription (STAT) (Kao, et al., 2011) and nuclear factor-kappa B (NF-κB)(Jang, et al., 2008).

Luteolin is naturally occurring in green plants, herbs and seeds (Middleton, et al., 2000), while the richest source of methoxyluteolin is in black ginger (*Kaempferia parviflora*) (Toda, et al., 2016). The structurally related 7, 8-dihydroxyflavone was shown to have BDNF-like activity (Jang, et al., 2010b) that is important for promoting neuronal survival and synaptic plasticity. In fact, loss of the gene encoding methyl-CpG binding protein in Rett syndrome, most patients of which also have ASD, has been correlated with decreased blood and CSF levels of BDNF (Katz, 2014;Li and Pozzo-Miller, 2014) and with autistic-like behavior in mouse models (Schaevitz, et al., 2010;Scattoni, et al., 2013;Li and Pozzo-Miller, 2014). Treatment of these mice with 7,8-dihydroxyflavone improved autistic-like behavior (Tsai, 2012;Johnson, et al., 2012). In contrast, increased levels of serum BDNF have been reported in some patients with ASD (Kasarpalkar, et al., 2014;Wang, et al., 2015;Bryn, et al., 2015;Zheng, et al., 2016b), especially in females (Spratt, et al., 2015), while other studies indicated no significant changes in peripheral BDNF levels (Rodrigues, et al., 2014;Bryn, et al., 2015). These findings suggest that a

dysregulation in BDNF signaling and/or its receptor function is present in ASD (Theoharides, et al., 2015a).

Interestingly, microglia release BDNF (Yuan, et al., 2010) and lack of microglial function, especially phagocytosis (M2 activation phenotype) has also been reported in Rett syndrome (Maezawa, et al., 2011;Zachariah, et al., 2012;Derecki, et al., 2013;Tsai, 2012;Derecki, et al., 2012). Luteolin globally alters the transcriptome of murine microglia to result in an anti-inflammatory phenotype (Dirscherl, et al., 2010) for phagocytosis. Luteolin has been shown to promote neuron survival (Zhu, et al., 2011) and improve learning/memory in mice (Jang, et al., 2010a). Luteolin has also been shown to reverse autism-like behavior in mice (Parker-Athill, et al., 2009) subjected to maternal immune activation (Hsiao, et al., 2012;Garay, et al., 2013). Luteolin (Dajas, et al., 2003;Nabavi, et al., 2015) and the related flavonoids 4′-methoxyflavone and 3′,4′-dimethoxyflavone (Fatokun, et al., 2013) are considered neuroprotective.

The allosteric inhibitors of mTOR, including rapamycin (sirolimus) and its analog, everolimus have been tried for the treatment of tuberous sclerosis, an autosomal dominant genetic disease with TSC1/2 mutations that accounts for 3-4 % of ASD with some success in treating seizures and behavioral deficits (Hwang, et al., 2016); however, their penetrance through the BBB is relatively poor. Furthermore, they preferentially inhibit mTORC1 rather than mTORC2 and have differential effects on the S6K and 4EBP1 downstream signaling arms, which regulate protein translation and cell proliferation (Choo, et al., 2008). Moreover the immunosuppresive properties of these inhibitors pre-dispose patients to infections, while acute renal failure has also been observed in some TSC1/2 patients treated with everlimous (Curatolo, et al., 2016).

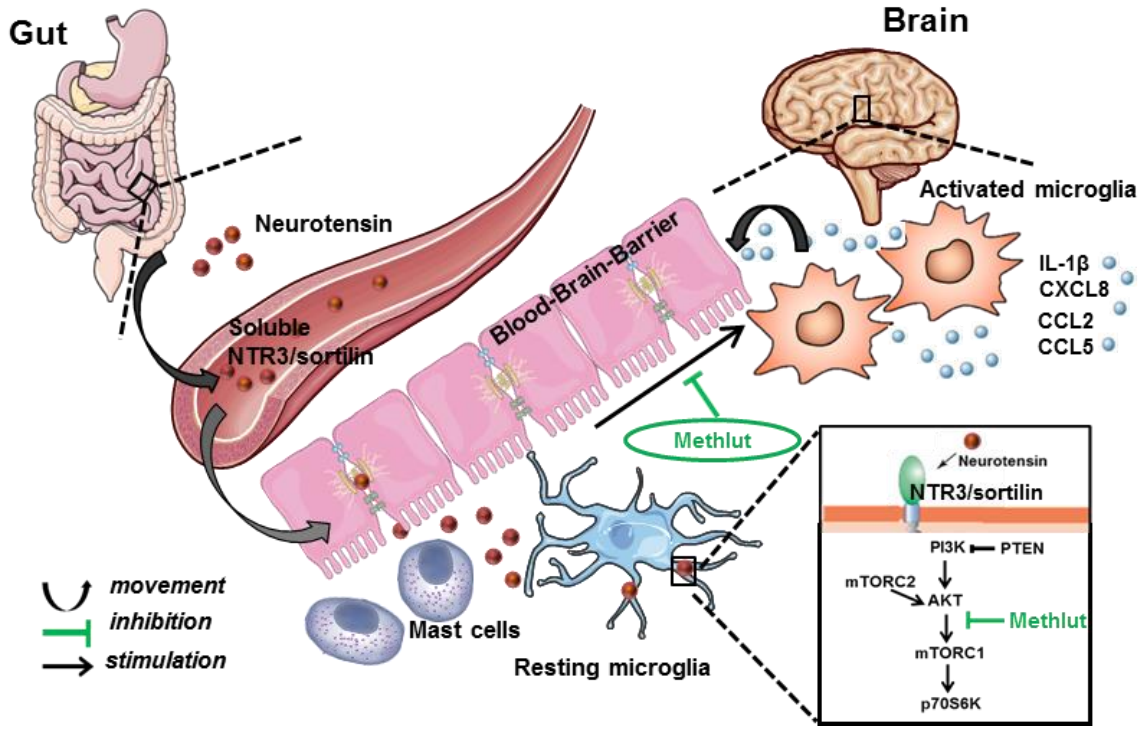


Figure 6.1. Proposed model by which NT may contribute to inflammation of the brain in ASD and using methoxyluteolin to inhibit these processes. Diagrammatic representation of how serum NT could derive primarily from the gut and increase permeability of the intestinal lumen and the BBB by stimulating perivascular mast cells. NT in the brain could then stimulate microglia via NTR3/sortilin, which is elevated in the serum of children with ASD. Pro-inflammatory mediator release from microglia through activation of mTOR signaling kinase, thus may contribute to inflammation of the brain and the pathogenesis of ASD. The flavonoid methoxyluteolin (Methlut) inhibits these processes and could be a novel treatment of ASD.

A luteolin-containing dietary formulation was reported to have beneficial effects in reducing ASD symptoms (Theoharides TC, et al., 2012a;Taliou, et al., 2013), and could even protect against memory loss (Theoharides, et al., 2015b) without adverse effects. Luteolin is generally safe (Formica and Regelson, 1995;Kawanishi, et al., 2005;Harwood, et al., 2007;Li, et al., 2010), and can even protect against drug-induced toxicity (Domitrovic, et al., 2009;Domitrovic, et al., 2013). Methoxyluteolin could

provide a superior therapeutic over luteolin for ASD and other diseases with inflammation of the brain. This is because it is a more potent inhibitor of microglia, MC and mTOR activation, then the available allosteric mTOR inhibitors and is metabolically more stable than luteolin (Walle, 2007), permitting for greater absorption and entry into the brain.

The several new findings presented in this report increase our understanding of the mechanistic pathway by which the peptide NT may play an important causal role in the pathogenesis of ASD. The data presented also provide a link between NTR3/sortilin to the pathological findings of microglia activation and inflammation found in brains of patients with ASD. Presently, there is no clinically effective drug to treat the pathophysiology of ASD (Ghosh, et al., 2013; Ruggeri, et al., 2014; Willsey and State MW, 2015). Targeting NTR3/sortilin and/or using methoxyluteolin may provide important novel therapeutic approaches for ASD.

6.2. PI3K/mTOR pathway: Therapeutic Target for MC Related-inflammatory Disorders.

Our present findings indicate that PI3K/AKT/mTOR activation is critical for the NT- or SP-stimulation of human MC and for the TNF-stimulation of human keratinocytes, resulting in the increased gene expression and release of pro-inflammatory mediators. The interactions among neuropeptides, MC and keratinocytes could be important for the pathophysiology of numerous inflammatory skin disorders, especially psoriasis (Scholzen, et al., 1998;Paus, et al., 2006;Theoharides, et al., 2004a;Theoharides, et al., 2016b). The elevated expression of mTOR and its phosphorylated substrates has also been reported in human psoriatic lesional skin (Choi, et al., 1997;Buerger, et al.,

2013;Chamcheu, et al., 2016). Moreover, psoriatic lesional skin is also positive for the presence of MC and its derived mediators (Toruniowa and Jablonska, 1988;Ackermann and Harvima, 1998;Jiang, et al., 2001), implying MC activation. Thus, targeting inhibition of MC and keratinocyte activation, as well as their interaction could be an effective therapy for psoriasis.

Importantly, the dual inhibition of the hyper-active PI3K/mTOR signaling components could ameliorate the abnormal activation of keratinocytes (Raychaudhuri and Raychaudhuri, 2014) and human MC (Blatt, et al., 2012). The increased mTOR gene expression is also evident in patients with systemic mastocytosis (Furumoto, et al., 2011; Smrz, et al., 2012; Theoharides, et al., 2015d). It has been suggested that selectively targeting mTOR complexes could effectively reduce proliferation of human MC associated with inflammation and MC disorders (Smrz, et al., 2011). Although, rapamycin had already been shown to inhibit the survival of KIT D816V mutated MC, leading to the constitutively active KIT receptor and mTOR (Gabillot-Carre, et al., 2006), a finding in about 80 % of all patients with systemic mastocytosis (Erben, et al., 2014;Hoermann, et al., 2014), treatment of which in patients using its clinically available analog everolimus resulted in organ toxicity (Parikh, et al., 2010).

Rapamycin had been tried for the treatment of psoriasis (Ormerod, et al., 2005), but yielded poor bioavailability and low efficacy (Paghdal and Schwartz, 2007), while the related analog, everlimous is currently in clinical trials for psoriasis (Frigerio, et al., 2007;Wei and Lai, 2015). However, many of these patients are prone to infections and several adverse effects, strangely including severe skin rashes, (Macdonald, et al., 2015) and acute renal failure (Curatolo, et al., 2016). Hence, there is an urgent need for

developing safe and effective dual inhibitors of the PI3K/mTOR pathway. Our present findings of mTOR activation in neuropeptide-induced human MC activation or TNF-stimulated human keratinocyte activation, and its inhibition by methoxyluteolin has clear therapeutic potential.

6.3. ERM Activation: Possible Molecular Target for Inhibition of MC Mediator Release

ERM are versatile proteins that serve as intramolecular scaffolds to regulate cytoskeleton rearrangement (Algrain, et al., 1993;Bretscher, 1999;Tsukita and Yonemura, 1999;Tsujita and Itoh, 2015) and as signal transducers, thereby regulating cellular processes such as cell adhesion, cell motility and mediator trafficking (Bretscher, et al., 2002;Fehon, et al., 2010;Neisch and Fehon, 2011), all implicated in MC activation. Our findings now indicate the importance of ERM in the regulation of human MC mediator secretion and provide a mechanistic insight in the activation of ERM. We report that ezrin and moesin are involved in the regulation of MC degranulation and for *de novo* synthesized release of TNF, VEGF and CCL2 in response to either allergic (IgE/anti-IgE) or peptide (SP) stimulation. Our findings add to the previous report, indicating impotance of ezrin in the regulation of FcεRI-mediated MC allergic responses (Staser, et al., 2013). We also report that activation of moesin, indicated by the increased levels of pmoesinThr588 levels is diminished in the presence of PI3K/mTOR inhibitors or the flavonoids luteolin and methoxyluteolin, indicating its regulatory role in MC activation.

The numerous MC-derived pro-inflammatory mediators, including pre-formed histamine, proteases and TNF, as well as *de novo* synthesized chemokines and cytokines are not only implicated in allergic or inflammatory processes, but also in the progression

of cancer (Theoharides and Conti, 2004). In fact, ERM facilitate tumor progression in variety of cancers, where either protein deficiency or mislocalization affects cell adhesion and motility, altering signals from growth factors and impairing the formation of receptor complexes (McClatchey, 2003;Clucas and Valderrama, 2014). In addition, moesin has recently been associated with higher risk of ASD; for instance, a study reported elevated levels of a non-coding RNA, encoded by the opposite strand of the moesin pseudogene 1, within postmortem cerebral cortex tissues of ASD patients that could down-regulate moesin (Kerin, et al., 2012). Moreover, low moesin protein is also evident in fetal brains of Down syndrome (Lubec, et al., 2001), which could affect neurodevelopmental processes. Suppression of moesin (Paglini, et al., 1998) or other ERM could hinder neuronal growth and alter morphology (Ramesh, 2004).

Our findings reported here provide some understanding of the regulation of human MC activation by ezrin and moesin and other signaling pathways that could be involved in the progression of neurodevelopmental disorders, cancer metastasis and inflammatory disorders. Targeting ERM, possibly with methoxyluteolin, may provide important novel therapeutic approaches for treatments of these conditions.

6.4. Methoxyluteolin: Potential Inhibitor of Inflammatory Responses

The increased pro-inflammatory gene expression and release of the following mediators: (a) IL-1β, CXCL8, CCL2 and CCL5 from microglia stimulated by NT or LPS, (b) TNF, CXCL8 and VEGF from MC stimulated by NT or SP and (c) IL-6, CXCL8 and VEGF from human keratinocytes stimulated by TNF was shown to be dependent on activation of PI3K/AKT/ mTOR. Importantly, methoxyluteolin attenuated mTOR signaling and significantly inhibited gene expression and release of all the pro-

inflammatory mediators, and hence the aberrant cellular activation. We also show that ERM regulate human MC mediator release and speculate that kinases downstream of mTORC2 may promote ERM activation, also inhibitable by methoxyluteolin (Fig. 6.2).

Methoxyluteolin could target multiple signaling proteins involved in microglia, MC and keratinocyte stimulation involving pro-inflammatory mediator gene expression, protein synthesis and release by: (a) ligand binding and MC stimulation via disruption of surface receptors for activation of FcεRI or GPCRs, (b) inhibition of calcium influx (Weng, et al., 2015), (c) gene expression via PI3K, mTORC1 (Kim, et al., 2008b) and nuclear transcription targets [nuclear factor-kappa B (NF-κB) (Dan, et al., 2008) or signal transducer and activator of transcription (STAT) 3] (Laplante and Sabatini, 2013;Saleiro and Platanias, 2015), (d) mediator production via mTORC1 involved in protein translation (Dibble and Cantley, 2015;Howell and Manning, 2011), (e) mediator secretion via mTORC2 for cytoskeleton rearrangement (Laplante and Sabatini, 2012), (f) induction of phosphatases for ERM dephosphorylation at the C-terminal sites (Staser, et al., 2013), (g) specific target proteins involved in vesicle fusion, such as soluble N-ethylmaleimide-sensitive factor attachment proteins (SNARE complexes) (Yang, et al., 2013;Yang, et al., 2015) and lastly (h) Ca^{2+} ATPase (Ogunbayo, et al. 2008; Agrawal AD, 2011) and/or Na^{+}, K^{+} ATPases (Mirsalikhova and Pakudina , 1977; Umarova, et al., 1998) for which the polyhrdroxylation of flavones (luteolin >> methoxyluteolin) is important for inhibition (Fig. 6.2).

Methoxyluteolin, a more potent inhibitor than luteolin or rapamycin for microglia, MC and keratinocyte activation via inhibition of PI3K and/or mTOR, thus providing a

unique therapeutic potential for the treatment of various inflammatory and MC disorders (Castells, 2006;Theoharides, et al., 2015b;Theoharides T.C., et al., 2016b).

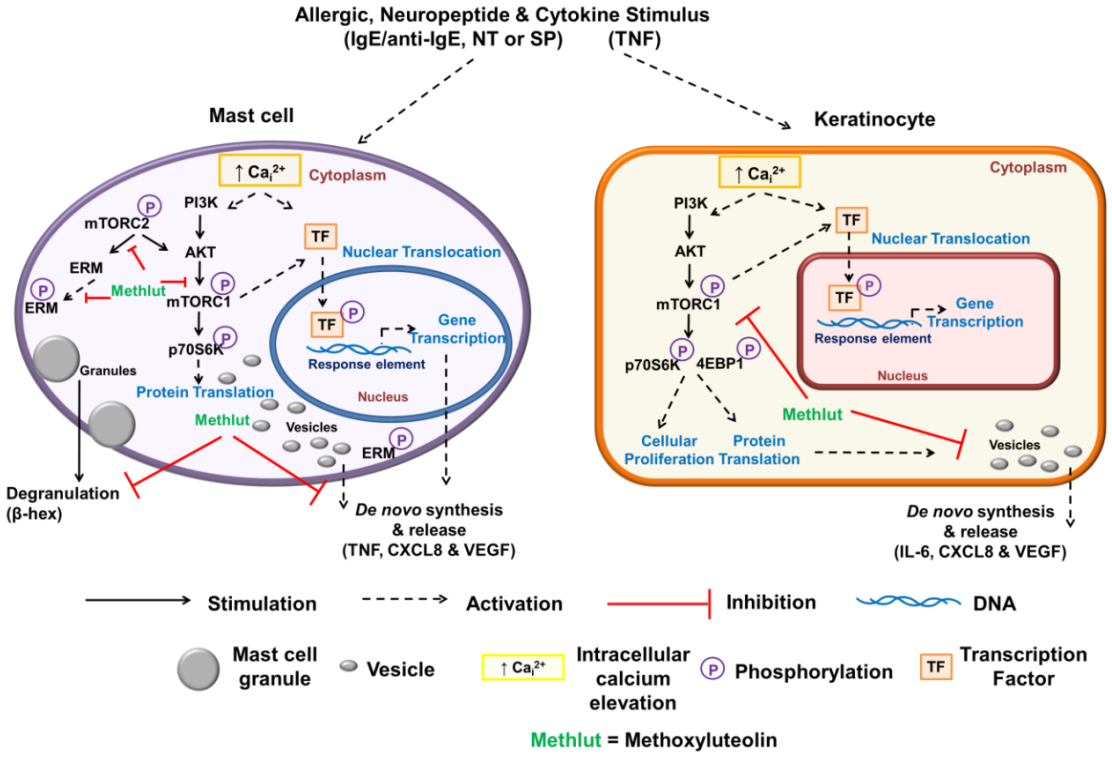


Figure 6.2. Proposed model of action of the flavonoid methoxyluteolin for inhibition aberrant human MC and keratinocyte responses. The neuropeptides NT and SP stimulate pro-inflammatory TNF, CXCL8 and VEGF gene expression and release from human MC, while the MC-derived cytokine, TNF stimulates pro-inflammatory IL-6, CXCL8 and VEGF gene expression and release from human keratinocytes. The activation of PI3K/AKT/mTOR signaling mediates these responses, as indicated by the increased levels of phosphorylated mTOR (pmTORSer2448) and the mTORC1 substrate, p70S6K (pThr389) in NT or SP stimulated MC. Similarly, the increased levels of these and phosphorylation of the second mTORC1 substrate, 4EBP1 (pThr37/46) is denoted; indicating activation of mTORC1 signaling in TNF stimulated keratinocytes. The flavonoid methoxyluteolin (methlut) more potently than luteolin or the allosteric mTOR inhibitor rapamycin decreases pro-inflammatory gene expression, possible via targeting inhibition of PI3K, mTORC1 (Kim, et al., 2008b) and the transcription factors downstream of mTOR signaling, which include NF-κB (Dan, et al., 2008) or STAT3 (Laplante and Sabatini, 2013;Saleiro and Platanias, 2015). Methlut also inhibits pro-inflammatory mediator production via mTORC1 involved in protein translation (Dibble and Cantley, 2015;Howell and Manning, 2011) in stimulated MC and keratinocytes. In addition, methlut targets inhibition of mTORC2 implicated in cytoskeletal rearrangement and blocks ERM activation, to decrease neuropeptide stimulated MC mediator release.

These include mastocytosis (Metcalfe and Akin, 2001;Theoharides, et al., 2015d;Petra AI, et al., 2014), asthma (Galli and Tsai, 2012;Sismanopoulos, et al., 2013), atopic dermatitis, (Vasiadi M, et al., 2012) and psoriasis (Sismanopoulos, et al., 2013;Maurer, et al., 2003;Theoharides, et al., 2012b).

Methoxyluteolin could be superior to luteolin not only because it is more potent inhibitor of microlgia, MC and keratinocyte activation, but it is metabolically more stable (Walle, 2007) due to its increased methylation , allowing for greater absorption in the skin (Ratz-Lyko, et al., 2015) and movement across the gut-brain-barrier or the BBB (Koirala, et al., 2016). Since, flavonoids have low oral bioavailability (~10 %) (Kawabata, et al., 2015; Lila, et al., 2016), utilizing novel drug delivery systems, such as liposomal formulations could enhance absorption. Orally administered flavonoids have been reported to enter the brain (Youdim, et al., 2004), but delivery through intranasal administration, as in the case of curcumin (Wang, et al., 2012; Chen, et al., 2013; Subhashini, et al., 2013) may bypass the issue of oral absorption and hepatic metabolism. Thus, methoxyluteolin may provide important novel therapeutics for ASD and for the dual inhibition of abnormally activated MC and keratinocytes in psoriasis and inflammatory skin disorders.

6.5. Future Studies

It is known that NT receptors in the human brain transiently increase after birth (Zsurger, et al., 1992) and are more concentrated in the hypothalamus (Najimi, et al., 2014) and the amygdala (Lantos, et al., 1996), which regulate behavior associated with the symptomatology of ASD. NTR3/sortilin, originally isolated from the human brain (Petersen, et al., 1997) is mainly expressed in the CNS during embryonic development (Hermans-Borgmeyer, et al., 1999), suggesting its importance in neurodevelopmental processes and microglia. To expand the findings presented here of the elevated and positively-correlated serum NT and NTR3/sortilin levels in patients with ASD, it will be interesting to determine the serum and cerebrospinal fluid (CSF) levels of NT and NTR3/sortilin from the same ASD patients. These studies will indicate whether the source of circulating serum NTR3/sortilin could originate from the resident-brain cells, such as neurons or microglia and enter the periphery via the disrupted BBB. It will also be important to determine if there is any diurnal variation in the release of NT or NTR3/sortilin.

To directly link the elevated serum NTR3/sortilin in patients with ASD to the pathological findings of microglia activation and inflammation of the brain, it will be important to investigate the expression of NT and its receptors in ASD brains from deceased patients and matched controls. Our laboratory has recently obtained frozen ASD and control brain tissues of male children (3-14 years) from the NIH NeuroBiobank. These specific regions include: (a) cerebral cortex/ Brodmann areas 10, 8, 9 46-47) and hippocampus, which regulates learning and stereotypic behavior, (b) amygdala that controls emotional behavior and (c) Broca area/ Brodmann areas 44 and 45, which

regulates language. Hence, future studies will include an investigation of the gene (by qRT-PCR) and protein (by Western blot) expression of NT receptors (NTR1, NTR2, NTR3/sortilin) and microglial-specific proteins (Iba1, CD11b and TREM2) or MC-specific proteins (tryptase and histidine decarboxylase). Subsequent studies will determine co-localization of specific NT receptors with either microglial or MC proteins by double-labeled immunohistochemistry and *in situ* tissue hybridization methods. These studies will be done in collaboration with Dr. Sabina Berretta (Associate Professor of Psychiatry/Neuroscience & Scientific Co-Director of the Harvard Brain Tissue Resource Center, McLean Hospital).

Activation of microglia and MC, as well as mTOR signaling in control and ASD brain regions described above can further be assessed by measuring the gene (qRT-PCR) and protein expression (Western blot or immunohistochemistry) of mTOR signaling components. NT and NTR3/sortilin, as well as activation of mTOR in the brains of ASD patients could implicate NT as the physiological stimulus for microglia or MC in ASD. Additional studies using co-cultures of human microglia and MC, and antagonists to neutralize the potential triggers, involved in cross-talk between microglia and MC, as shown in Table 1.1 will identify crucial mediators that may stimulate these immune cells in auto/paracrine feed-back loops, resulting in their aberrant immune cell activation. Identification of the unique mediators that promote cross-talk between microglia and MC, which is now considered important in inflammation of the brain (Skaper, et al., 2012;Skaper, et al., 2013;Skaper, et al., 2014b;Dong, et al., 2014b) could provide additional molecular targets for inhibition and ASD treatment. Moreover, subsequent

studies could screen for these mediators in serum and CSF of patients with ASD and serve as potential biomarkers for ASD diagnosis.

It will also be interesting to decipher the specific roles of ezrin and moesin and their regulation of 'phospho-cycling' at specific sites in human MC activation by allergic or peptide stimuli, and inhibition by methoxyluteolin. To expand on the present findings that implicate the PI3K/mTOR pathway in mediating moesin phosphorylation at Thr588, which is associated with active protein conformation, our laboratory will further investigate the corresponding phosphorylation patterns on Thr567 in ezrin and Thr564 in radixin. It will also be important to determine the specific kinase, downstream of mTORC2, which include the Rho family (Matsui, et al., 1998;Hall, 2012), PKCα and PKCθ (Pietromonaco, et al., 1998;Bretscher, et al., 2002) that is responsible for phosphorylation of ERM in stimulated MC. These studies will provide a molecular mechanism for the regulation of human MC activation by ezrin and moesin.

Previously, our laboratory had identified the inhibitory phosphorylation signals induced by the MC stabilizer cromolyn (Correia, et al., 1996;Wang, et al., 1999) and flavonoids at several sites on moesin (Ser56/74 and/or Thr66) within the FERM domain of moesin, which were also associated with the inactivation of the calcium-insensitive PKCθ (Kempuraj, et al., 2005). It is possible that both luteolin and methoxyluteolin could induce phosphorylation at these inhibitory sites in human MC, and also activate phosphatases to dephosphorylate the C-terminal Thr sites on ezrin and/or moesin to inhibit MC activation. Hence, employing mass spectrometry analysis or site-directed mutagenesis to identify these inhibitory sites, and generating new phospho-antibodies for these, will allow the determination of ERM phosphorylation patterns in regulation of

microglial, MC and keartinocyte secretion. These studies will provide additional information on the molecular mechanisms involved for action of methoxyluteolin for inhibition of aberrant cellular responses.

Here, we report that keratinocyte pro-inflammatory mediator gene synthesis and release in response to TNF stimulation, a relevant stimulus that could derive from MC (Ackermann and Harvima, 1998) and is implicated in psoriasis (Olivieri, et al., 2009;Sedger and McDermott, 2014;Grine, et al., 2015), is mediated via PI3K/mTOR activation. The expression of phosphorylated mTOR and its downstream substrates S6K has also been shown to be elevated in lesional-psoriatic skin (Choi, et al., 1997;Buerger, et al., 2013;Raychaudhuri and Raychaudhuri, 2014). Thus, future studies could investigate the activation of PI3K/mTOR signaling and MC and keratinocytes (using specific marker proteins and derived pro-inflammatory mediators) in lesional- psoriatic and non-lesional punch biopsies obtained from Athens Hospital, Greece.

Determining the expression of specific MC-derived mediators such as histamine or tryptase, and keratinocyte-derived mediator growth-regulated oncogene or keratins, as well as activation of mTOR in the psoriatic skin will increase understanding of MC and keratinocyte the pathophysiological interactions, resulting in cutaneous inflammation. Identifying novel molecular targets of inhibition by methoxyluteolin for microlgia, MC and keratinocyte activation and proliferation, including other kinases, phosphatases, transcription targets and cytoskeletal or transmembrane proteins will add to our current mechanistic knowledge and spur the development of new therapies to treat inflammatory diseases. Lastly, formulation of methoxyluteolin in vehicles (e.g. liposomes) for targeted delivery to cells/tissues of interest would be important.

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Appendix

List of Publications

1. **Patel AB**, Tsilioni I, Leeman SE and Theoharides TC. Neurotensin stimulates NTR3/sortilin and mTOR in human microglia: Potential therapeutic target in autism inhibitable by methoxyluteolin. *PNAS, In press, 2016*

2. **Patel AB** and Theoharides TC. Neurotensin and substance P stimulation of TNF, CXCL8 and VEGF release from human mast cells involves mTOR activation, inhibitable by tetramethoxyluteolin. *Mol Pharm, Submitted, 2016*

3. **Patel AB** and Theoharides TC. Regulation of degranulation and *de novo* synthesized mediator release from human mast cells by the cytoskeletal ezrin and moesin**.** *JPET, Submitted, 2016*

4. **Patel AB**, Tsilioni I and Theoharides TC. TNF and IL-17 stimulate pro-inflammatory IL-6, CXCL8 and VEGF secretion from human keratinocytes via mTOR activation, also detectable in psoriatic skin, and inhibited by the methoxyluteolin. *JID, In preparation, 2016*

5. Theoharides TC, Tsilioni I, **Patel AB** and Doyle R. Atopic diseases and inflammation of the brain in the pathogenesis of autism spectrum disorders. *Transl Psychiatry,6:e844*

6. Tellechea A, Leal EC, Kafanas A, Auster ME, Kuchibhotla S, Ostrovsky Y, Tecilazich F, Baltzis D, Zheng Y, Carvalho E, Zabolotny JM, Weng Z, Petra A, **Patel A**, Panagiotidou S, Pradhan-Nabzdyk L, Theoharides TC, Veves A. Mast cells regulate wound healing. *Diabetes,65:2006-19*

7. Weng Z, **Patel AB**, Panagiotidou S, Theoharides TC. 2014; The novel flavone tetramethoxyluteolin is a potent inhibitor of human mast cells. *J Allergy Clin Immunol, 135:1044-52*

8. Weng Z, **Patel AB**, Vasiadi M, Therianou A, Theoharides TC. 2014 Luteolin inhibits human keratinocyte activation and decreases NF-κB induction that is increased in psoriatic skin. *PLoS One, 9: e90739*

9. Theoharides TC, Asadi S and **Patel AB**. Focal brain inflammation and autism. *J. Neuroinflammation,10:46*