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Anti-inflammatory effects of tick salivary protease inhibitors on mouse microglial cells

Bachelor's thesis

YIFAN MAYR

Supervisor: Jindřich Chmelař RNDr. Ph.D

Department of Medical Biology

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Annotation

This project evaluated the effect of several tick salivary protease inhibitors on inflammation induced in BV-2 microglial cells, by assessing levels of inflammation mediators and cell adhesion.

Affirmation

I hereby declare that I have worked on the submitted bachelor's thesis independently. All additional sources are listed in the bibliography section.

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Yifan Mayr

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Abstract

Neurodegenerative diseases (ND) such as Alzheimer's and Parkinson's have become a pressing threat to human health – Alzheimer's disease alone is predicted to affect 48 million people by 2026. Although the causes of these diseases are still under rigorous investigation, it is well accepted that chronic neuroinflammation is a major contributor. Microglia, the resident immune cells in the brain and the commanders of neuroinflammation, are found to be excessively activated in ND. One potential therapeutic strategy to treat ND is therefore to inhibit microglial activation, hence relieving neuroinflammation.

Among the many tested inducers of microglial activation are proteases. Their ability to stimulate inflammation in microglia makes protease inhibitors possible anti-inflammatory agents. Various protease inhibitors have been reported to possess immunosuppressive abilities. Particularly, protease inhibitors in tick saliva have been found to undermine host's immunity. In this project, I investigated the effect of several tick salivary protease inhibitors on activated microglia.

To simulate the inflammatory state of microglia, inflammation inducers were used. Activated microglia were treated with several protease inhibitors of interest (cystatins G1, G2, G9, G16, serpins IRS-2, S8K, and sialostatins L1 and L2). The severity of inflammation was assessed according to the levels of nitric oxide, cytokines, and cell adhesion.

Interestingly, cystatins G1 and G2 repeatedly enhanced the production of inflammation mediators. Other protease inhibitors, however, did not follow a consistent trend of effects. Adhesion of microglia to laminin, fibronectin and plastic (polystyrene) was in accordance with published data.

Our limited understanding of the tested protease inhibitors may have led to inaccurate assumptions about their roles; further experimentation would be necessary. Nevertheless, protease inhibitors remain a promising class of molecules for treatment of ND.

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Introduction

1. Neuroinflammation and Microglia

In mice and humans alike, leukocytes are the guards and effector cells of the immune system, patrolling and defending the organism against pathogens. The defense system is vast and intricate: the adaptive immune system has B lymphocytes to secrete antibodies against specific pathogens, and T lymphocytes to either eliminate the infected cells directly or summon other cells for the deed; the innate immune system uses neutrophils and other granulocytes to destroy invading bacteria via various mechanisms, and macrophages to phagocytize microorganisms, foreign agents and damaged senescent cells (Alberts, 2015). The delicate interplay between the innate and adaptive immune systems deploys countless cells and processes to maintain homeostasis in the body. It is indispensable to our well-being.

However, due to the highly selective blood-brain barrier insulating the central nervous system (CNS) from the circulating blood, leukocytes cannot penetrate into the CNS (Ballabh et al., 2004). Even though the barrier protects the CNS from external threats to a large extent, some immune defense is necessary for the body's most crucial organ. Microglia, together with astrocytes, oligodendrocytes, and ependymal cells are glial cells in the CNS (Figure 1), which provide support and ensure proper functioning of neurons (Campbell, 2009). While astrocytes exchange substances between neurons and blood capillaries, oligodendrocytes insulate axons with myelin sheath, and ependymal cells line the cavities filled with cerebrospinal fluid, microglia are the immune cells responsible for defense and repair in the CNS (Wake et al., 2011).

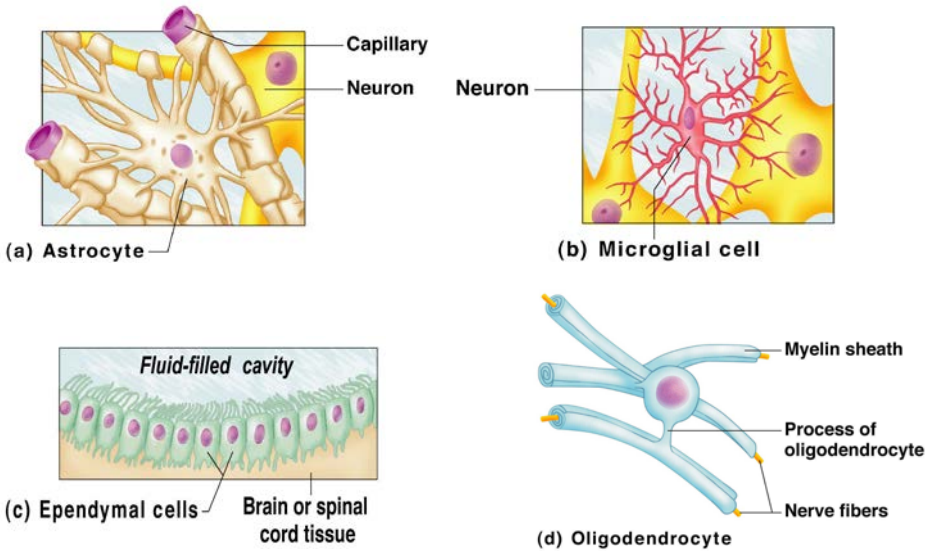

Figure 1. Glial cells in the CNS. (Campbell, 2009)

In a healthy brain where no imminent threat is present, microglia are in a "resting" state, taking a ramified shape while randomly patrolling the brain parenchyma with their processes (Nimmerjahn et al., 2005). Their motility allows them to quickly respond to pathological conditions such as neuroinflammation, brain injuries, exposure to toxins, etc. Depending on the stimulus, microglia can adopt different activated phenotypes with different functions (Cherry et al., 2014). Although still debatable (Ransohoff, 2016), one commonly used system – transferred from microglia's close relative macrophages – categorizes the activation phenotypes into M1 and M2 (Michelucci et al., 2009). In the classical activation model M1, microglia processes rapidly extend to the sites in need and initiate downstream signaling including the nuclear factor κB (NF-κB) pathway (Schlachetzki et al., 2013) to secrete pro-inflammatory mediators such as nitric oxide (NO), tumor necrosis factor (TNF)-α and interleukin (IL)-6 (Prinz & Priller, 2014). The alternative activation M2 is anti-inflammatory (Gordon, 2003).

Depending on the stimuli and function, M2 can be subdivided into M2a the alternative activated phenotype and M2c the deactivated phenotype. M2a, induced by IL-4 or IL-13, functions to suppress inflammation and enhance phagocytosis. M2c, stimulated by IL-10, glucocorticoids, or TGF-β, is involved in tissue remodeling and wound repair after the inflammation has been suppressed (Cherry et al., 2014).

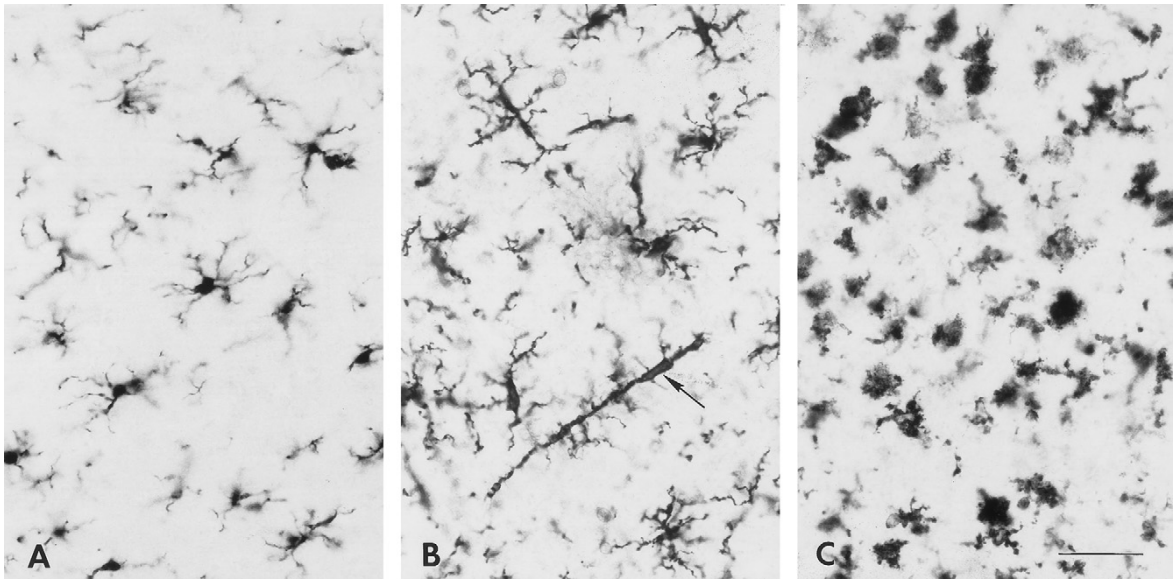

Figure 2. Human brain microglial cells. A) Resting microglia in normal brain. B) Activated microglia in diseased cerebral cortex have thicker processes and larger cell bodies. Arrows point to rod cells. C) Microglia in regions of frank pathology transform into phagocytic macrophages. Scale bar = 40 μm. (Squire, 2013)

The different phenotypes are interchangeable. It has been observed that during acute brain injuries, microglia first become M1 to remove invading pathogens and dead cells, then transform to the M2 phenotype for clean-up and repair (Kigerl et al., 2009). If the transformation is not successful and microglia remain in the M1 state, the secreted pro-inflammatory mediators trigger further microglia activation and thus a self-amplifying loop begins. Chronic inflammation can cause more cell death, tissue destruction, synapse loss and eventually cognition disruption (Cherry et al., 2014).

The pathology of neuroinflammation is therefore of great therapeutic importance and various cell culture models have been developed and used to study neuroinflammation. Highly analogous to primary microglial cells but easier to culture, the BV-2 microglia cell line, isolated from mouse strain C57BL/6 and immortalized by using a retrovirus J2 carrying the *v-myc* oncogene (Blasi et al., 1990), is a commonly used model.

2. Inflammation Inducers – Lipopolysaccharide LPS and Interferon-gamma IFN-γ

To simulate the environment in the CNS after injury or invasion, pro-M1 inflammation inducers are used to stimulate microglia. LPS and IFN-γ are two known pro-M1 inducers (Michelucci et al., 2009).

LPS, an endotoxin found in the cell wall of Gram-negative bacteria (Alberts, 2015), effectively stimulates microglial cells (Cheong et al., 2011). It comprises a hydrophobic head called lipid A, which anchors to the outer membrane of the cell wall, a non-repeating core of oligosaccharide, and a chain of repeating polysaccharides called O-antigen (Raetz & Whitfield, 2002). LPS is recognized as a pathogen-associated molecular pattern (PAMP) by the pattern recognition receptors (PRRs) of the innate immune system. Toll-like receptor TLR-4, a type of PRR, recognizes LPS (Alberts, 2015) and initiates a cascade of inflammatory responses including excretion of nitric oxide, TNF-α, and IL-6 in BV-2 microglial cells (Gold et al., 2014). Research has shown that most cytokine release after exposure to LPS occurs within 24 hours (Papageorgiou et al., 2016).

IFN-γ is used as a co-stimulator with LPS in microglial inflammation studies (Schroder et al., 2004). It is produced by natural killer (NK) cells, natural killer T (NKT) cells of the innate immune system, and by CD4+ T helper cell type 1 (Th1) lymphocytes, CD8+ cytotoxic lymphocytes, and professional antigen-presenting cells (APCs) if adaptive immunity is established (Schroder et al., 2004). IFN-γ enhances the stimulating effect of LPS by augmenting TLR-4 expression on the cell surface and increasing LPS binding ability.

3. Cytokines – TNF-α and IL-6

One major characteristic of the classical microglia activation phenotype M1 is the elevation of pro-inflammatory cytokine levels (Prinz & Priller, 2014). An increased production of TNF-α and IL-6 in microglial cells upon stimulation with LPS has been well documented in many studies (Cheong et al., 2011; Dong et al., 2014; Gold et al., 2014).

TNF-α causes hemorrhagic necrosis of tumors in animals, hence the name tumor necrosis factor. It is a homotrimeric molecule (Figure 3), with molecular weight of each monomer around 17kDa (Eck & Sprang, 1989). Following the detection of an injury or infection, microglia produce TNF-α to recruit more microglia or astrocytes, leading to stronger inflammation. Chronic neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease lead to the secretion of TNF-α as well, due to the amyloid-β peptides and aggregated synuclein, respectively (Cheng et al., 2014). TNF-α is known to be cytotoxic and contributes to programmed cell death.

The normal physiological concentration of IL-6 in human serum is around 1–5 pg/ml (Hunter & Jones, 2015), but increases upon stimulation, reaching the µg/ml range in extreme cases such as a meningococcal septic shock (Waage et al., 1989). The low basal level and rapid elevation upon stimulation qualifies IL-6 as a marker for inflammation (Hunter & Jones, 2015).

Mature mouse IL-6 contains 187 amino acids arranged in alpha helices (Figure 4), and weighs around 21 kDa (Van Snick et al., 1988). It is a functional homolog of the human IL-6 with 42% protein homology (Van Snick et al., 1988). Once released, IL-6 binds to the receptor IL-6R, and they together associate with gp130, a signal-transducing subunit. Dimerization of these complexes then activates downstream pathways, including the mitogen-activated protein kinase MAPK cascade (Hunter & Jones, 2015). IL-6 is also found to promote macrophage differentiation and TNF receptor expression (Hunter & Jones, 2015). Interestingly, when secreted in skeletal muscles in response to exercise, IL-6 acts as an anti-inflammatory myokine (Febbraio & Pedersen, 2005).

Both TNF-α and IL-6 can cross the blood-brain barrier, but with different transporters (Banks et al., 1994). Transport rates are affected by disease state and injuries (Quan & Banks, 2007).

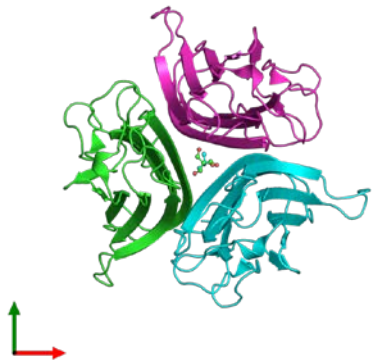

Figure 3. Top view of a mouse TNF-α homotrimer at 1.4Å resolution. Two ligands are bound: 2-amino-2-hydroxymethyl-propane-1,3-diol and isopropyl alcohol. (Eck & Sprang, 1989)

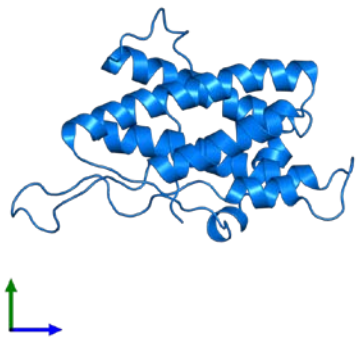

Figure 4. Front view of a mouse IL-6 monomer. (Veverka et al., 2012)

4. Neurodegenerative Diseases and Microglia

Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and many other neurodegenerative diseases have become household names due to their widespread presence in the aging population. Until 2006, Alzheimer's disease alone had affected 24 million people and the number is expected to double every twenty years (World Health Organization, 2006).

While the causes and mechanisms of neurodegeneration remain largely unknown, evidence of anti-inflammatory drugs delaying development of PD and AD suggests that neuroinflammation is the culprit (Cunningham, 2013). As research has shown, microglia-mediated neuroinflammation causes cell damage, a prominent characteristic of neurodegenerative diseases (Cherry et al., 2014).

Alzheimer's disease, often marked by loss of memory and cognitive function, is associated with neuropathological changes such as the formation of beta amyloid (Aβ) plaques and neurofibrillary tangles of tau protein, which leads to neuron death and synapse loss (Serrano-Pozo et al., 2011). In addition to its neurotoxicity, Aβ activates microglia through toll-like receptors, increases production of amyloid precursor proteins and Aβ cleavage enzymes – all propagating a vicious cycle of continuous chronic inflammation (Cherry et al., 2014).

Although microglia are capable of an M2 phenotype, which removes Aβ via phagocytosis, this ability declines as the disease advances. It has been reported that microglia switch from M2 to M1 in aged mice models of Alzheimer's disease (Jimenez et al., 2008), which may have resulted from lower sensitivity of IL-4Rα (Fenn et al., 2012).

Parkinson's disease is characterized by loss of dopaminergic neurons and aggregation of Lewy bodies, which are composed of alpha-synuclein. In a familiar scheme, alpha-synuclein and microglia form a loop of inflammation, feeding each other with constant stimuli (Sanchez-Guajardo et al., 2015). It has been documented that alpha-synuclein activates the toll-like receptors on microglia (Roodveldt et al., 2013) and induces the production of nitric oxide, which can lead to cell death of nearby neurons through nitration of alpha-synuclein (Shavali et al., 2006).

5. Proteases in Neuroinflammation

Proteases are enzymes which catabolize proteins by hydrolyzing peptide bonds between amino acids. Depending on the nucleophile used for proteolysis, proteases can be categorized into six classes: aspartic, glutamic, metallo, cysteine, serine, and threonine proteases (Lopez-Otin & Bond, 2008). The serine proteases, for example, have a serine as their nucleophilic enzymatic site. Chymotrypsin, trypsin and elastase are serine proteases synthesized in the pancreas and transported to the small intestine to perform their digestive duties (Lehninger et al., 2005).

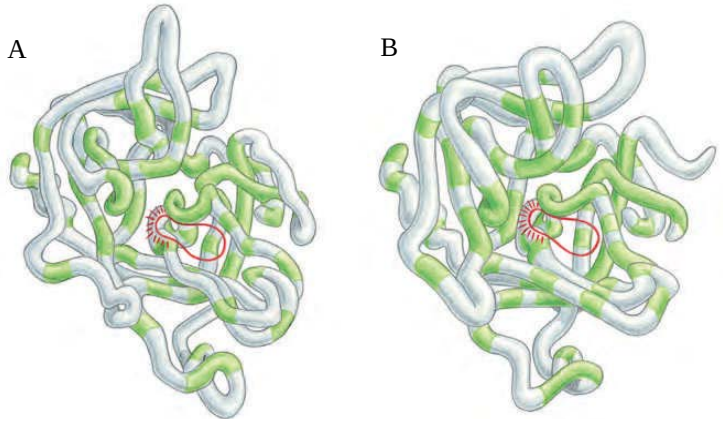

Figure 5. Structures of elastase (A) and chymotrypsin (B). Common amino acids are marked in green; the active site is marked in red.

In addition to digestion, processes which depend on proteolysis include protein regulation, molecule modification, inflammation, and phagocytosis (Lopez-Otin & Bond, 2008). Despite the versatile roles they play, proteases retain similar structures within the same family (Figure 5) – most of their tertiary protein structures match (Alberts, 2015). Several serine proteases from immune cells are especially important in the scope of this project. For example, granzyme A in cytotoxic lymphocytes induces the release of pro-inflammatory cytokines, granzyme B, H, K and M are involved in apoptosis (Heutinck et al., 2010); elastase, cathepsin G (cat-G) and proteinase 3 (PR3) from neutrophils are linked to inflammation and tissue damage during bacterial infection (Heutinck et al., 2010); chymase and tryptase from mast cells promote cytokine production. Neutrophil elastase is also involved in inflammatory disorders such as chronic obstructive pulmonary disease (COPD) and inflammatory bowel disease (IBD). Plasmin, once formed from its inactive precursor plasminogen, is capable of stimulating the production of inflammation mediators such as cytokines and reactive oxygen species (ROS). It also induces chemotaxis of monocytes,

increasing immune responses in the region, which can be detrimental in chronic inflammatory diseases (Syrovets et al., 2012).

The mechanism through which cytokine production is regulated can be connected to serine proteases activating protease-activated receptors (PARs), which are involved in neuroinflammation and neurodegenerative diseases (Shpacovitch et al., 2008). In fact, all four PARs have been identified in the brain. Microglia, specifically the BV-2 cell line, express mRNA for PARs (Balcaitis et al., 2003), indicating that microglia are subject to activation by proteases.

Among the four PARs identified so far, PAR_2 is activated by trypsin/tryptase, and the other three are sensitive to thrombin, a serine protease (Gingrich & Traynelis, 2000). Activation of PARs by thrombin initiates downstream pathways, including the MAPK pathway which enhances gene expression.

The relevance of proteases to neuroinflammation does not stop there: after the injection of PAR_1-activating peptides (APs), synthetic peptides capable of activating PAR_1, astrocytes and microglia are activated and local inflammation is induced (Shpacovitch et al., 2008). It was also discovered in mouse models of multiple sclerosis that astrocytes and microglia with higher PAR_2 contribute to demyelination of neurons and thus neurodegenerative processes (Shpacovitch et al., 2008).

6. Protease Inhibitors in the Regulation of Neuroinflammation

The activating effect of proteases on microglia allows protease inhibitors to be potential regulators of neuroinflammation. Serpins and cystatins, inhibitors of serine proteases and cysteine proteases, respectively, play an important role in regulating cytokine production and phagocytosis (Chmelar et al., 2017).

Protease nexin (PN1), a potent serpin, is naturally present in astrocytes and has repeatedly shown neuroprotective abilities (Cooley et al., 2001; Gingrich & Traynelis, 2000; Pawlinski et al., 2004). Neuroserpin, a serine protease inhibitor in neurons, suppresses the serine protease tissue plasminogen activator (tPA) (Gingrich & Traynelis, 2000). A study using knock-out mice models found that inhibition of thrombin by hirudin, combined with the absence of PAR_2, reduces inflammation and LPS-induced mortality (Pawlinski et al., 2004).

SerpinB1, produced by neutrophils and macrophages, efficiently suppressed neutrophil elastase, PR3 and Cat-G, and prevented self-inflammation in the cells (Cooley et al., 2001).

In contrast to serpins, cystatins are reversible inhibitors of cysteine proteases such as papaya protease I and legumains (Kordis & Turk, 2009). Cystatins inhibit, for example, lysosomal cathepsins, which are involved in protein degradation and apoptosis (Turk et al., 2012). Antigen degradation via major histocompatibility II (MHCII) by lymphocytes, inhibition of interleukin-1 converting enzyme (ICE) (caspase-1), which is required for the release of inflammatory cytokines IL-1β and IL-18 (Chen et al., 2014), and inflammasome regulation, all attribute to cystatins (Jin & Flavell, 2010).

Given the importance of serpins in inflammation control, it is comprehensible that polymerization (therefore unavailability of functional neuroserpins) is a partial cause of familial dementia (Davis et al., 1999). In addition, a 2007 study found that the level of neuroserpin in cerebrospinal fluid is higher in Alzheimer's patients than in age-matched control groups (Nielsen et al.).

Several cystatins have been reported to be involved in neurodegenerative diseases (Kaur & Levy, 2012). Cystatin A (CysA) and cystatin B (CysB) have been found in large quantities in senile plaques in Alzheimer's patients (Bernstein et al., 1996). Absence of CysB resulted in progressive myoclonic epilepsy type 1 in mice, but the symptoms, e.g. motor incoordination, neuronal loss, and gliosis, were alleviated by overexpression of CysC (Kaur et al., 2010).

The ability of serpins and cystatins to effectively suppress the activity of serine and cysteine proteases presents them as potential novel therapeutic treatments for neuroinflammatory and neurodegenerative diseases.

7. Tested Tick Protease Inhibitors and Their Function

Most of the time, survival in nature is a constant tug of war. When a host is attacked by ticks, its immune system works to minimize the damage by, for example, releasing cytokines (Brossard & Wikel, 2004). As ticks' survival depends on their blood meals, it is not a surprise that tick saliva contains molecular effectors, e.g. protease inhibitors, which alter the host's immune system (Chmelar et al., 2017) to maximize the effect of an attack. Some of

the protease inhibitors tested in this project have been reported on their immunosuppressive abilities, while others are still under investigation.

7.1. Tick cystatins G1, G2, G9, G16

Among the possible candidates tested for anti-inflammatory effects on microglia BV-2 cells were four cystatins from tick species *Ixodes ricinus* – G1, G2, G9, and G16. All were recombinant proteins made by the research group led by Michail Kotsyfakis from the Institute of Parasitology of Biology Centre, CAS.

7.2. Tick serpin S8K

A tick salivary serpin with the code name "S8K" was tested as well. The recombinant protein used in this project was provided by the research group led by Michail Kotsyfakis from the Institute of Parasitology of Biology Centre, CAS.

7.3. Tick serpin IRS-2

Ixodes ricinus serpin-2 (IRS-2), fittingly, is an exogenous salivary serpin in the tick species *Ixodes ricinus* which has a tryptophan as its cleavage site. Its anti-inflammatory effect is well documented. An *in vivo* study using mouse paw edema models showed that IRS-2 reduced edema inflammation in mouse paws and neutrophil recruitment into inflamed regions (Chmelar et al., 2011). It has also been reported that IRS-2 inhibits mast cell chymase and cathepsin G, reduces levels of pro-inflammatory cytokine IL-6 in dendritic cells, and suppresses maturation of pro-inflammatory T helper 17 cells (Th17) by disrupting the IL-6-dependent JAK/STAT3 pathway (Palenikova et al., 2015). Platelet aggregation caused by cathepsin G and thrombin is also suppressed by IRS-2 (Chmelar et al., 2011).

7.4. Sialostatins L1, L2

Sialostatin L1 (Figure 6) from the tick species *Ixodes scapularis* is a cystatin with demonstrated anti-inflammation ability (Valenzuela et al., 2002). It inhibits cathepsins L, V, C, X, S, and papaya protease I, with a stronger effect on cathepsin L (Kotsyfakis et al., 2006). Proliferation of cytotoxic T lymphocytes and neutrophil myeloperoxidase activity in carrageenan-induced paw edema are reduced by L1 as well (Kotsyfakis et al., 2006). L1 decreases production of cytokine IL-2 and IL-9 (Horka et al., 2012) possibly by suppressing

T lymphocytes proliferation (Kotsyfakis et al., 2006), and production of IL-12 and TNF-α by inhibiting maturation of their producer dendritic cells (Sa-Nunes et al., 2009). Dendritic cell maturation can be inhibited by L1 limiting cathepsin S, a cysteine protease which is involved in antigen-presentation (Sa-Nunes et al., 2009), or by L1 disrupting IFN-β-triggered JAK/STAT signaling (Lieskovska et al., 2015). IFN-β production by Borrelia or TLR-7 ligand-activated dendritic cells is also lowered (Lieskovska et al., 2015).

Figure 6. Crystal structure of L1 at 2.68 Å resolution. PDB: 4ZM8 (Kotsyfakis et al., 2010)

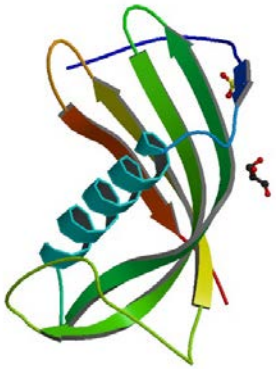

Figure 7. Crystal structure of L2 at 1.8 Å resolution. PDB: 3LH4 (Kotsyfakis et al., 2010)

Sialostatin L2 (Figure 7) has the same origin as L1 and a similar sequence. In addition to a preferential affinity to cathepsins L and V, L1 works with cathepsins S and C. Infection by Gram-negative bacteria *A. phagocytophilum* can be suppressed by L2 through indirect inhibition of caspase-1, an enzyme required for maturation of pro-inflammatory cytokines IL-1β and IL-18 (Chen et al., 2014). Similar to L1, L2 hinders the JAK/STAT signaling in dendritic cells as well (Lieskovska et al., 2015). An *in vivo* study (Kotsyfakis et al., 2008) showed that guinea pigs immunized with L2 were less susceptible to exposure to *I. scapularis* nymphs – smaller tick size, reduced number of eggs, higher rejection rate and longer time required for a blood meal compared to control groups.

8. Regulating Pro-inflammatory Cytokine Levels as a Therapeutic Strategy

The solid scientific evidence strongly supports the link between microglial activation and the pathogenesis of neurodegenerative diseases such as Alzheimer's and Parkinson's. The potential of anti-inflammatory agents to treat neuroinflammation-mediated disorders is well recognized and plenty of research has been invested into identifying possible candidates. In fact, a wide variety of substances has been reported to reduce the production of pro-inflammatory cytokines in activated BV-2 microglial cells, among others are a water extract

of the root of the herb *polygala tenuifolia* (Cheong et al., 2011), the protease inhibitor α1-antitrypsin (Gold et al., 2014), thymoquinone (Taka et al., 2015), and lithium (Dong et al., 2014).

However, very limited information is available regarding the effect of tick protease inhibitors on microglia – a search in NCBI PubMed with the keywords "tick microglia inflammation" returned merely 3 results, none of which described the role of tick protease inhibitors on microglia inflammation. My work intends to contribute to filling the gap of knowledge by evaluating the anti-inflammatory effects of selected tick salivary protease inhibitors on mouse microglial cells.

Aim of the Thesis

This project aimed to test the inflammatory response of BV-2 microglial cells to ligands of Toll-like receptors and to evaluate the viability of several recombinant tick salivary protease inhibitors as anti-inflammatory agents on activated BV-2 microglial cells.

List of Materials

Name	Description	Source
BV-2 cells	Mouse microglial cells, C57BL/6	Trianafyllos Chavakis, TU Dresden
RPMI 1640 w/ L-Glutamine		BioSera
Antibiotic antimycotic		
Fetal Bovine Serum (FBS)		BioSera
Cell culture medium	10% FBS, 1% Antibiotic antimycotic in RPMI 1640 w/ L-Glutamine	
Bovine Serum Albumin (BSA)	30% solution	BioSera
Cell starvation medium	0.5% BSA, 1% Antibiotic antimycotic in RPMI 1640 w/ L-Glutamine	
Phosphate Buffered Saline (PBS)		Gibco
Trypan Blue stain 0.5%		Sigma Aldrich
Lipopolysaccharide (LPS)	LPS O111:B4	Sigma Aldrich
Interferon γ (IFN-γ)		Thermo Fisher
Dexamethasone		Sigma Aldrich
Griess Reagent modified		Sigma Aldrich
Tick salivary cystatins	G1, G2, G9, G16 from *Ixodes ricinus*	Produced by Jan Kotál and Michail Kotsyfakis
Tick salivary serpins	S8K, IRS2	Produced by Jan Kotál and Michail Kotsyfakis
Tick serpins (sialostatins) L1, L2	*Ixodes scapularis*	Produced Michail Kotsyfakis

ELISA Ready-SET-Go!		e-Bioscience
PBS-Tween	PBS, 0.05% Tween-20	
Stop solution	2N H_2SO_4	
HBSS solution	Diluted from 10x HBSS solution with DI water	Gibco
3% BSA	Low fatty acid BSA powder (Sigma Aldrich) dissolved in sterile PBS	
HBSS + 0.1% BSA	Low fatty acid BSA powder dissolved in HBSS solution	
Calcein	2mM stock	Sigma Aldrich
Collagen	1mg/mL stock	Sigma Aldrich
Laminin	1mg/mL stock	Sigma Aldrich
Fibronectin	200μg/mL stock	Sigma Aldrich
Phorbol 12-myristate 13-acetate (PMA)	1mg/mL stock	Sigma Aldrich

Experiments/methods

Cell Culture

BV-2 mouse microglial cells were cultivated according to a protocol from P. Romano, A. Manniello *et al.* (2009). Cells were seeded in T75 flasks in pre-warmed cell culture medium and maintained in a humidified incubator with 5% CO_2 at 37°C. The medium was changed every three or four days. Cultures were passaged at approx. 85% confluence by washing with PBS prior to re-seeding 10–20% of the cells. Cells were cultivated and used for experiments until passage 25.

Cell Growth Optimization

To determine the optimal timing and culture condition of preparing BV-2 cells for experiments, different amounts of cells were incubated in 5% CO_2 at 37°C for various lengths of time. Cells from confluent cultures were washed with pre-warmed PBS, an aliquot was stained 1:4 or 1:9 with Trypan Blue for counting, and the rest was diluted to a parent suspension at a concentration of 160,000 cells/well with cell culture medium. Different cell amounts were achieved by further diluting the parent cell suspension. Each cell amount was performed in triplicates. The cell growth/confluence was determined at various incubation time lengths under a light microscope.

NO Production

Levels of accumulated nitrite, a stable oxidized product of nitric oxide, were measured using Griess reagent following an established protocol for IC-21 cells.

BV-2 cell culture suspensions were centrifuged and re-suspended in the cell starvation medium to reach a concentration of 1 million cells/mL. 200µL of the cell suspension was seeded per well in a 96-well plate. After pre-incubation with inhibitors, inflammation stimulators such as LPS and IFN-γ were added. The cells were incubated for 24h in the incubator with 5% CO_2 at 37°C. 100µL supernatant was transferred to an absorbance reading plate, mixed with 100µL Griess reagent, incubated in the dark for 15min, and measured at 540nm using a Synergy™ H1 microplate reader (BioTek). Nitrite concentrations were calculated using an external standard calibration curve from known concentrations of sodium nitrite.

Cytokine Analysis by ELISA

BV-2 cells were harvested from confluent cell cultures, pre-incubated with inhibitors and stimulated with LPS. The levels of cytokines TNF-α, IL-6, and IL-1β were detected using Enzyme-Linked Immunosorbant Assay (ELISA). Concentrations were calculated from absorbance values at 450nm with 570nm as reference wavelength.

BV-2 cell culture suspensions were centrifuged and re-suspended in the cell starvation medium to reach a concentration of 160,000 cells/mL. 500µL of the cell suspension was seeded per well in a 24-well plate and incubated for 24h. The cells were starved for 4h, pre-incubated with tested inhibitors at a concentration of 1µM or 3µM for 1h, and then stimulated by 100ng/mL LPS for 24h. The supernatant was collected and stored at -20°C for further analysis.

Following the protocol of "ELISA Ready-SET-GO!" from eBioscience with minor modifications, 50µL of thawed cell supernatant was used for ELISA measurements. Absorbance was measured at 450nm with 570nm as a correction wavelength using a Synergy™ H1 microplate reader. The concentrations of the target cytokines were calculated according to the external standard calibration series.

Detailed procedure:

Corning Costar ELISA plates were coated with 50µL/well of capture antibody in coating buffer at 4°C over night, then blocked with 200µL assay diluent at room temperature for 1h. The wells were washed four times with PBS-Tween. 50µL standard solutions and samples were transferred to the plate and incubated at room temperature for 2h. The wells were washed four times with PBS-Tween. 50µL detection antibody was added and incubated at room temperature for 1h. The wells were washed four times with PBS-Tween. 100µL Avidin-HRP was added and incubated at room temperature for 30min. The wells were washed six times at 1min intervals. 100µL substrate solution was added and incubated at room temperature in the dark for 7~10min depending on the speed of color development. The reaction was stopped with 100µL 2N H_2SO_4 before measuring the absorbance.

Cell Adhesion

Based on an adhesion protocol for neutrophils, several experiments with different conditions were conducted to produce adequate responses.

A Costar 3590-96 well plate was coated with 50µL 3% BSA as a control and several extracellular matrix proteins – laminin, collagen and fibronectin – at the concentration of 10µg/mL. The plate was stored overnight at 4°C before being blocked with 50µL 3% BSA for 1h in an incubator with 5% CO_2 at 37°C. Subsequently the plate was washed three times with sterile PBS. The plate was stored filled with sterile PBS at 4°C.

11 million BV-2 cells were washed and re-suspended into 2mL HBSS + 0.1% BSA medium, 20µL of a fluorescent dye Calcein was added to final 2uM concentration and incubated for 20min in an incubator with 5% CO_2 at 37°C. The cells were then washed once with 2mL HBSS + 0.1% BSA medium and distributed to six tubes each containing 250µL cell suspension. The cells were pre-incubated with 2µM inhibitors for 1h in an incubator with 5% CO_2 at 37°C, followed by incubation with 50ng/mL PMA for 20min in an incubator with 5% CO_2 at 37°C. The cells were washed once with 2mL HBSS + 0.1% BSA medium before triplicates of 150µL of cell suspension were transferred to the wells in the prepared adhesion plate. The cells were incubated for 30min in an incubator with 5% CO_2 at 37°C. Before and in between washes of the plate with 200µL PBS, absorbance was measured with the Synergy™ H1 microplate reader at an emission wavelength of 535nm using an excitation wavelength of 490nm.

A slightly different protocol (protocol 2 in table 1) was adopted to evaluate the effect of PMA incubation time on adhesion rate, the differences are described in Table 1.

Table 1. Differences in adhesion protocols

	Protocol 1 (testing inhibitors)	**Protocol 2 (incubation time optimization)**
Medium	HBSS + 0.1% BSA	HBSS + 1% BSA
Initial cell count	11 million; 150,000 cells/well	13.5 million; 187,500 cells/well
Cell treatment	Calcein→wash→ inhibitors→PMA→ wash→measure	Calcein→wash→PMA→ measure
Activation time	20min	2h, 1h, 1/2h

Statistical Analysis

Results are presented as mean ± standard error of mean (SEM). Statistical significance was calculated using one-way analysis of variance (ANOVA) with Bonferroni *post-hoc* test, $p < 0.05$ was considered statistically significant. Data analysis was performed with the XL Toolbox add-in for Excel, version 7.2.10 (xltoolbox.sourceforge.net).

Results

Cell Growth Optimization

80,000 cells/well in a 24-well plate showed optimal growth in 24h. This configuration was adopted for further experiments in cytokine analysis with ELISA.

Table 2. Confluence of BV-2 cells cultivated in a 24-well plate. Approximately 85% confluence is considered optimal.

Growth time	No cells	5,000 cells/well	10,000 cells/well	20,000 cells/well	40,000 cells/well	80,000 cells/well
7h	0	6%	10%	25%	40%	70%
24h	0	8%	15%	40%	60%	85%
31h	0	10%	25%	60%	80%	95%
42h	0	15%	40%	65%	80%	95%
49h	0	20%	45%	70%	90%	95%

NO Production

BV-2 microglial cells were stimulated with 100ng/mL LPS and/or 100U/mL IFN-γ for 24h. Absorbance at 540nm should be directly proportional to the level of accumulated nitrite. Both LPS and IFN-γ increased the NO level in BV-2 cells, but not in synergy (Figure 8A). 500ng/mL of dexamethasone decreased the level of accumulated nitrite in BV-2 supernatant (Figure 8B). Preliminary screening of cystatins from *Ixodes ricinus* and sialostatins from *Ixodes scapularis* did not yield significant results, only G1 showed some inhibition, but the effect was not statistically significant (Figure 8C).

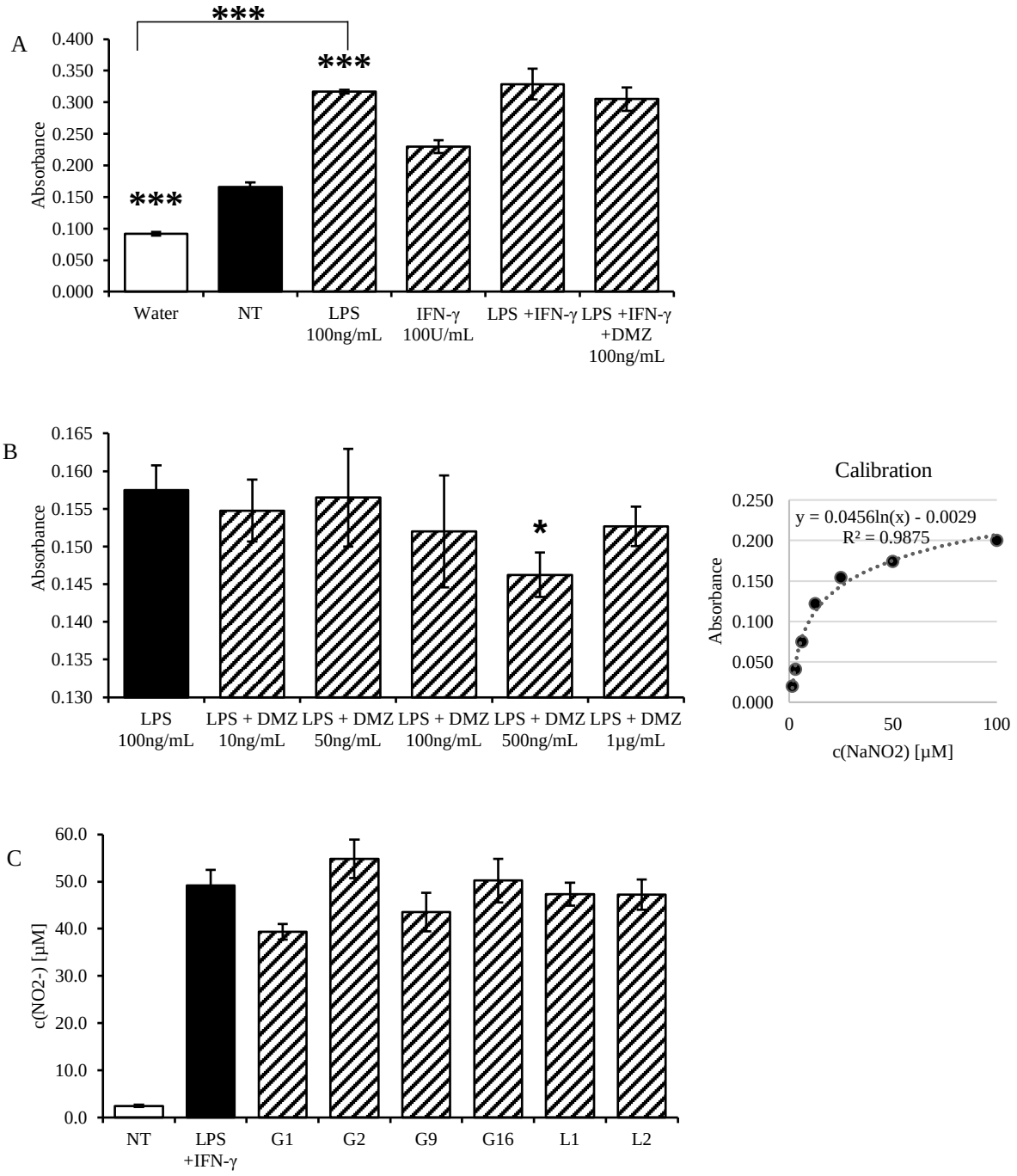

Figure 8. Effect of inhibitors on nitric oxide production. BV-2 cells were starved, pre-incubated with inhibitors, and stimulated with inflammation inducers. Cell culture supernatants after treatment were analyzed by using Griess reagent. Values are presented as mean ± SEM. NT = no treatment, LPS = 100ng/mL LPS, IFN-γ = 100U/mL IFN-γ. Statistical significance compared to the positive control group is marked. (* p-value <0.05, ** p-value <0.01, *** p-value <0.001). A) Cells were stimulated with 100ng/mL LPS and/or 100U/mL IFN-γ, and subsequently treated with 100ng/mL dexamethasone for 24h. n=4. B) Cells were pre-incubated with different concentrations of dexamethasone for 4h before stimulation with 100ng/mL LPS for 24h. n=3. C) Cells were pre-incubated with 3µM of inhibitors G1, G2, G9, G16, L1, and L2 for 2h, then co-stimulated with 100ng/mL LPS and 100U/mL IFN-γ for 24h. n= 3.

Cytokine Analysis by ELISA

As a preliminary test of the production of TNF-α, IL-6, and IL-1β in BV-2 cells, their levels in a negative control group and a positive control group were examined. In the positive control group, where the cells were stimulated with 100ng/mL LPS, both TNF-α and IL-6 levels increased (Figure 9), while IL-1β level was below zero in both groups when calculated from the calibration line (not shown).

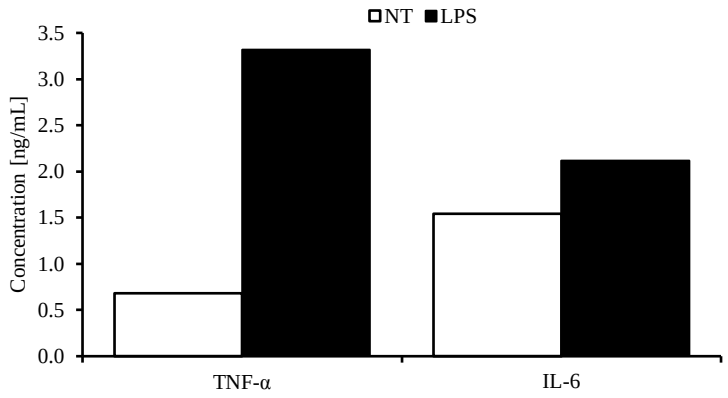

Figure 9. Levels of TNF-α and IL-6 in negative and positive control. BV-2 cells were starved for 4h, then stimulated with 100ng/mL LPS for 24h. NT = no treatment, LPS = 100ng/mL LPS, n=1.

Pre-incubation with inhibitors generated non-conclusive results: pre-incubation with G1 and G2 increased the IL-6 level in three separate experiments (Figure 10A, B, D); IRS2, S8K, and G9 reduced the IL-6 level slightly in one experiment (Figure 10B), but not in the others. The differences, however, were mostly not statistically significant compared to the positive control group (Figure 10). TNF-α level followed a similar trend in three experiments (Figure 11A, B, C), except in the fourth experiment (Figure 11D) where LPS induced a 20-fold increase in TNF-α production, and all inhibitors had a significant reducing effect on TNF-α level.

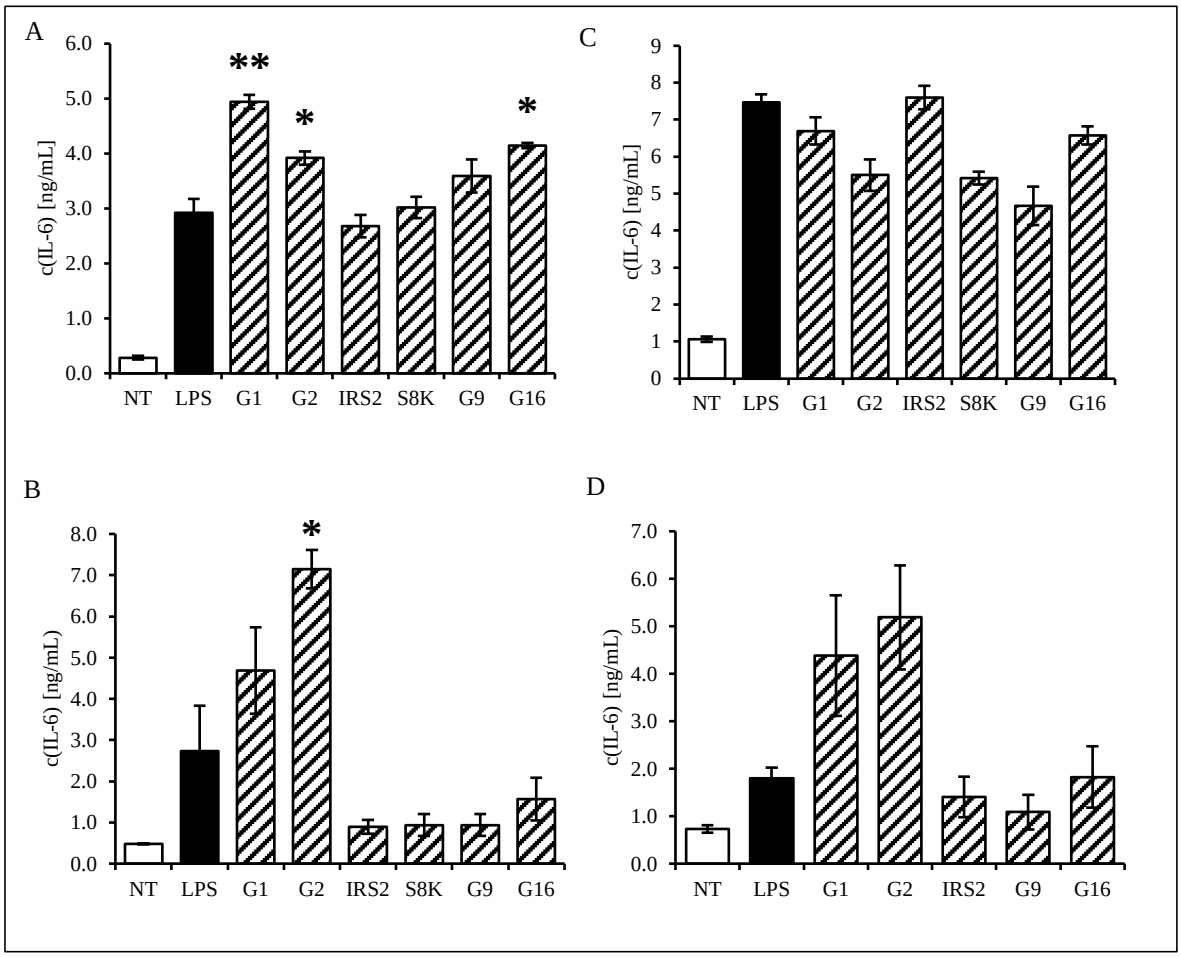

Figure 10. Effect of inhibitors on IL-6 level. BV-2 cells were starved, pre-incubated with 1µM of inhibitors for 1h, and stimulated with LPS for 24h. Cell culture supernatants after treatment were examined using ELISA. NT = no treatment, LPS = 100ng/mL LPS. Values are presented as mean ± SEM. n=3. Statistical significance compared to the LPS group is marked. (* p-value <0.05, ** p-value <0.01, *** p-value <0.001). A, B, C) Cells were pre-incubated with G1, G2, IRS2, S8K, G9, and G16. D) Cells were pre-incubated with G1, G2, IRS2, G9, and G16.

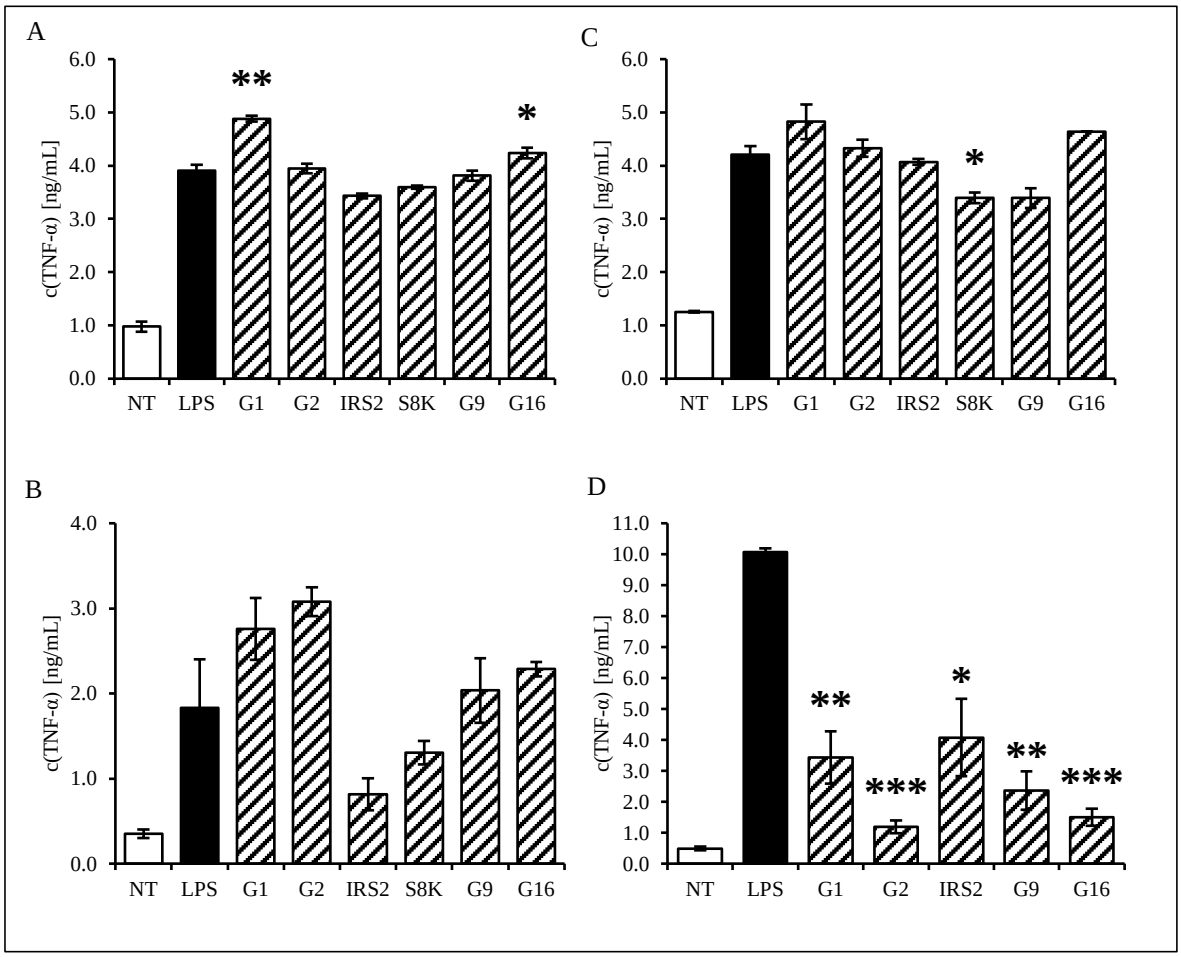

Figure 11. Effect of inhibitors on TNF-α level. BV-2 cells were starved, pre-incubated with 1µM of inhibitors for 1h, and stimulated with LPS for 24h. Cell culture supernatants after treatment were examined using ELISA. Values are presented as mean ± SEM, n = 3. NT = no treatment, LPS = 100ng/mL LPS. Statistical significance compared to LPS group is marked. (* p-value <0.05, ** p-value <0.01, *** p-value <0.001). A, B, C) Cells were pre-incubated with G1, G2, IRS2, S8K, G9, and G16. D) Cells were pre-incubated with G1, G2, IRS2, G9, and G16.

To illustrate a more direct view on the effect of inhibitors across experiments, cytokine level values from the same experiments (Figure 10, Figure ***11***A-C) were recalculated relative to the average concentration from the LPS group, which was considered as 100%. Each concentration value was divided by the average concentration from the LPS group and presented as a percentage:

$$\%\ of\ LPS = \frac{c(x)}{\bar{c}(LPS)} * 100\%$$

$c(x)$: Any concentration value $\bar{c}(LPS)$: Average concentration from the LPS group

Normalizing the concentration data to percentage values allows for a comparison across experiments. The combined data shows that both cystatins G1 and G2 significantly increased IL-6 production, and that G1 enhanced TNF-α levels in BV-2 cells.

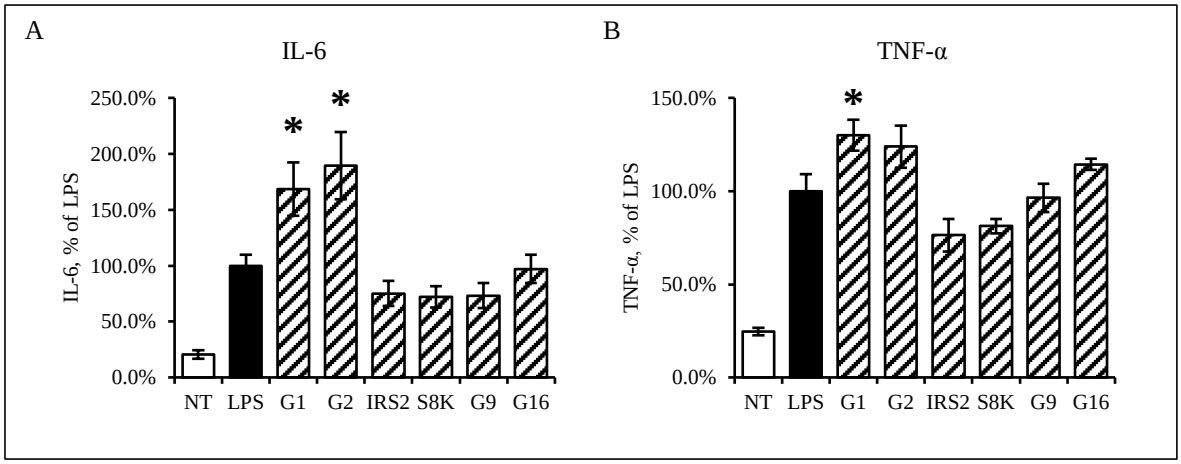

Figure 12. Effect of inhibitors on IL-6 and TNF-α level as percentage of the positive control. BV-2 cells were starved, pre-incubated with 1µM of inhibitors – G1, G2, IRS2, S8K, G9, and G16 – for 1h, and stimulated with LPS for 24h. Cell culture supernatants after treatment were examined using ELISA. NT = no treatment, LPS = 100ng/mL LPS. Values are presented as mean ± SEM. Statistical significance compared to the LPS group is marked. (* p-value <0.05, ** p-value <0.01, *** p-value <0.001). A) Values are from the same four IL-6 experiments described in Figure 10. n=9 for S8K, and n= 12 for all other groups. B) Values are from three TNF-α experiments described in Figure 11A-C. n=9.

Higher concentrations of inhibitors, namely 3µM of G1, G2, S8K, and G9, were also tested on BV-2 cells. All inhibitors enhanced the IL-6 production (Figure 13).

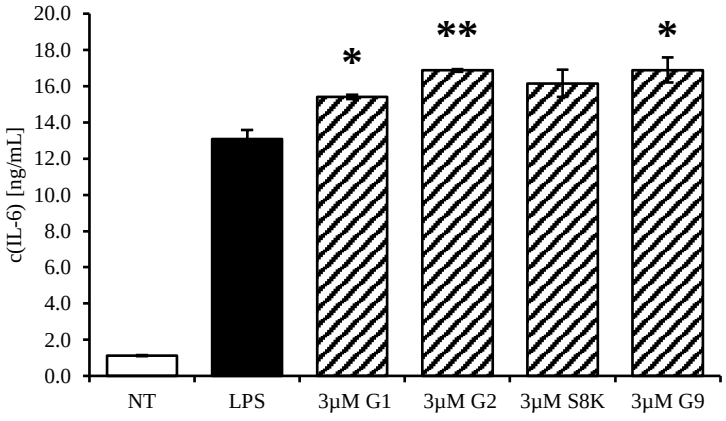

Figure 13. Effect of 3µM inhibitors on IL-6 level. BV-2 cells were starved, pre-incubated with 3µM or 1µM of inhibitors - G1, G2, S8K, and G9 - for 1h, and stimulated with LPS for 24h. Cell culture supernatants after treatment were examined using ELISA. Values are presented as mean ± SEM, n = 3. NT = no treatment, LPS = 100ng/mL LPS. Statistical significance compared to LPS group is marked. (* p-value <0.05, ** p-value <0.01, *** p-value <0.001).

A parallel experiment measuring TNF-α and IL-6 production was conducted with IC-21 cells to compare the immune responses from BV-2, the brain macrophages, and IC-21, the peritoneal macrophages. Similar to that in BV-2 cells, LPS induced a higher amount of excreted IL-6, and G1 enhanced IL-6 production even further (Figure 14A). TNF-α level did not change after LPS stimulation (Figure 14B).

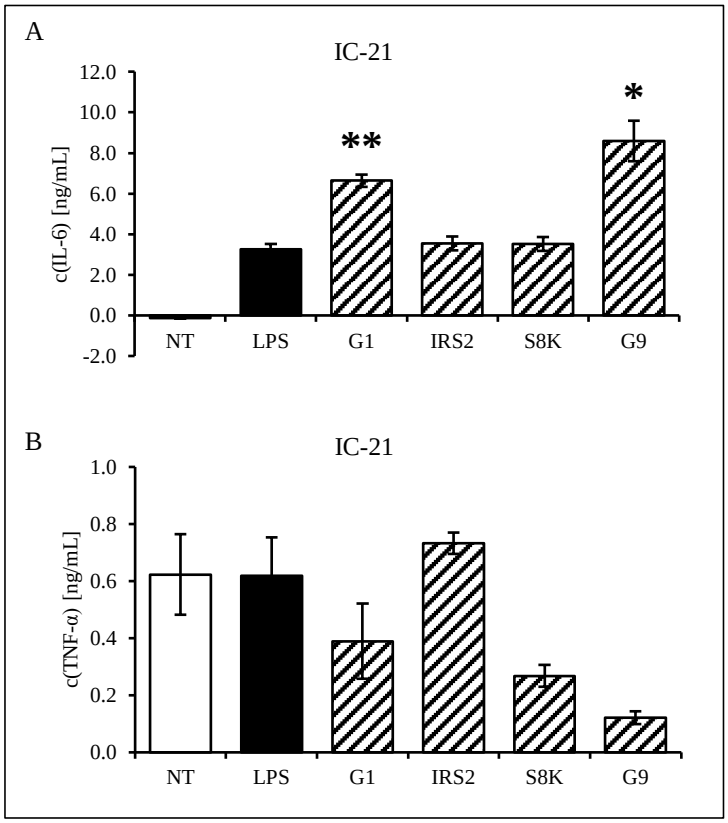

Figure 14. Effect of inhibitors on IL-6 and TNF-α levels. IC-21 cells were starved, pre-incubated with 1μM of inhibitors – G1, IRS2, S8K, and G9 – for 1h, and stimulated with LPS for 24h. Cell culture supernatants after treatment were examined using ELISA. Values are presented as mean ± SEM, n =3. NT = no treatment, LPS = 100ng/mL LPS. Statistical significance compared to LPS group is marked. (* p-value <0.05, ** p-value <0.01, *** p-value <0.001).

Cell Adhesion

Adhesion assays were performed on BV-2 cells in order to optimize experimental conditions that were based on a protocol for neutrophils. Adhesion is expressed as the percentage of cells remaining attached to the surface after three washes. 3% BSA and proteins of extracellular matrix, such as laminin, collagen and fibronectin, were coated on a 96-well plate as the adhesion surfaces.

In one experiment, PMA-stimulated BV-2 cells exhibited weaker adhesion to all substrates than the non-stimulated cells did (Figure 15), while in another experiment, PMA did not have an effect on adhesion rate (Figure 16). Treatment with G1 increased adhesion to all substrates (Figure 15). The replication of the same conditions reproduced a weaker adhesion-enhancing effect of G1 (Figure 16). Both experiments (Figure 15, Figure 16), however, did not display expected activation by PMA.

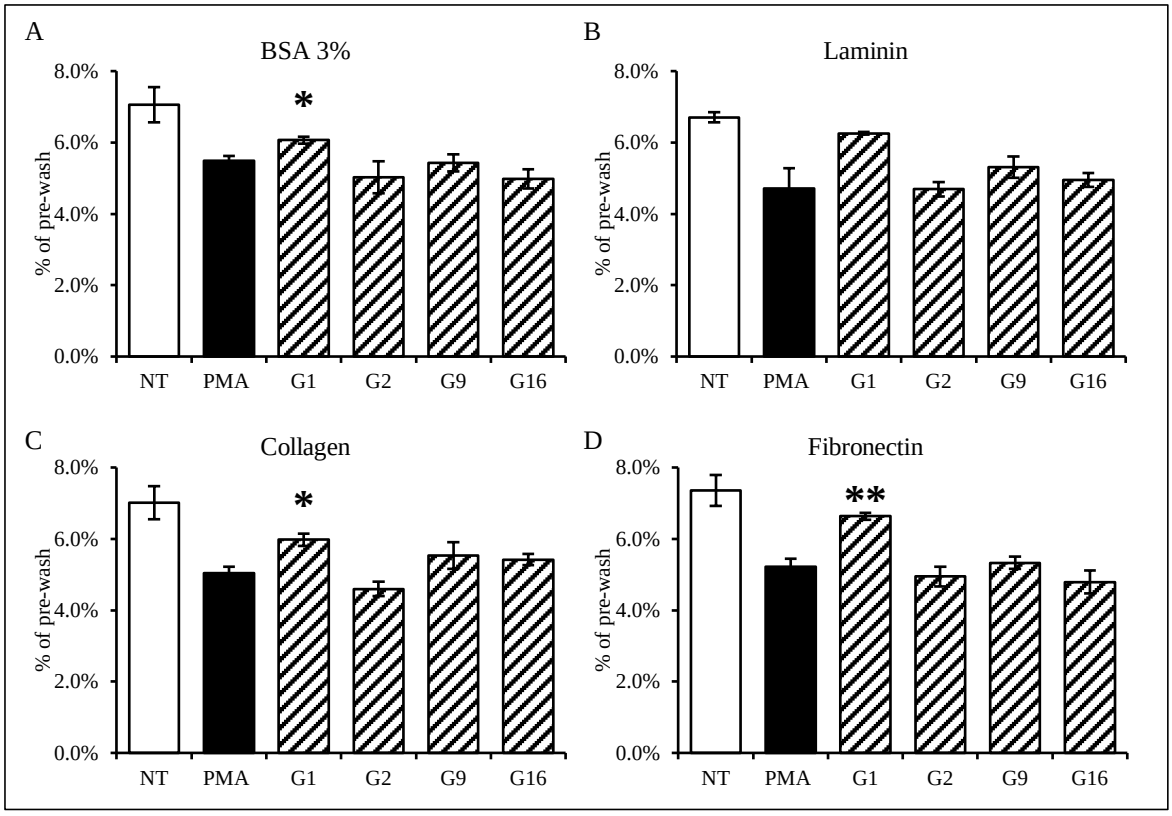

Figure 15. Effect of inhibitors on PMA-activated BV-2 adhesion after 3 washes. BV-2 cells were in HBSS + 0.1% BSA medium, labelled with fluorescent dye Calcein, washed 1x with medium, pre-incubated with inhibitors G1, G2, G9, G16 for 1h, activated by PMA for 20min, then washed again with medium before measurements. NT = no treatment, PMA = 50ng/mL. Values are presented as mean ± SEM. n=3. Statistical significance compared to the PMA group is marked. (* p-value <0.05). A) Plate coated with 50µL 3% BSA overnight. B) Plate coated with 50µL 10µg/mL laminin overnight. C) Plate coated with 50µL 10µg/mL collagen overnight. D) Plate coated with 50µL 10µg/mL fibronectin overnight.

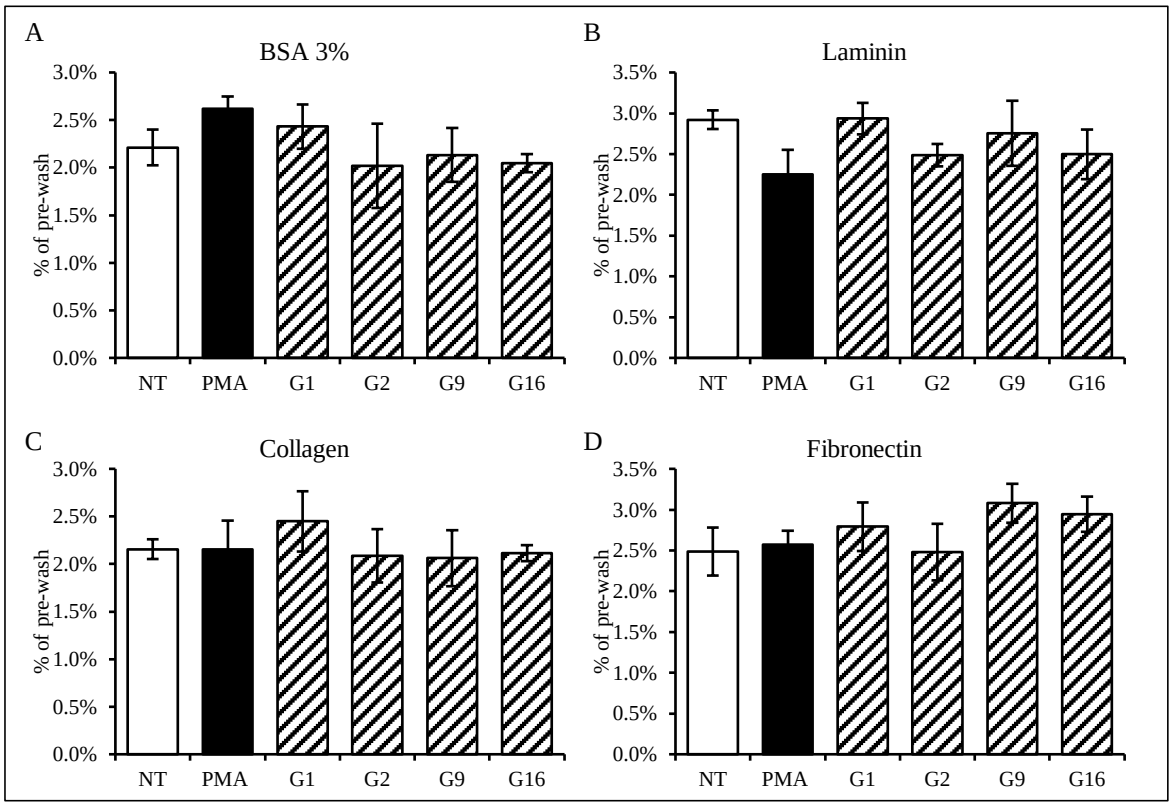

Figure 16. Effect of inhibitors on PMA-activated BV-2 adhesion after 3 washes. BV-2 cells were in HBSS + 0.1% BSA medium, labelled with 2µM fluorescent dye Calcein, washed 1x with medium, pre-incubated with inhibitors 2µM G1, G2, G9, G16 for 1h, activated by PMA for 20min, then washed again with medium before measurements. NT = no treatment, PMA = 50ng/mL. Values are presented as mean ± SEM. n=3. Statistical significance compared to the PMA group is marked. (* p-value <0.05). A) Plate coated with 50µL 3% BSA overnight. B) Plate coated with 50µL 10µg/mL laminin overnight. C) Plate coated with 50µL 10µg/mL collagen overnight. D) Plate coated with 50µL 10µg/mL fibronectin overnight.

An experiment on the effect of PMA on BV-2 adhesion was conducted with modified conditions. The cells were allowed to be activated in 50ng/mL PMA for 2h, 1h, and 1/2h before measurements. In Figure 17, it appears that PMA triggered higher adhesion across all incubation times, especially to BSA and fibronectin. BV-2 adhered strongly to cell culture plate surface (polystyrene) and fibronectin but poorly to laminin and collagen (Figure 17).

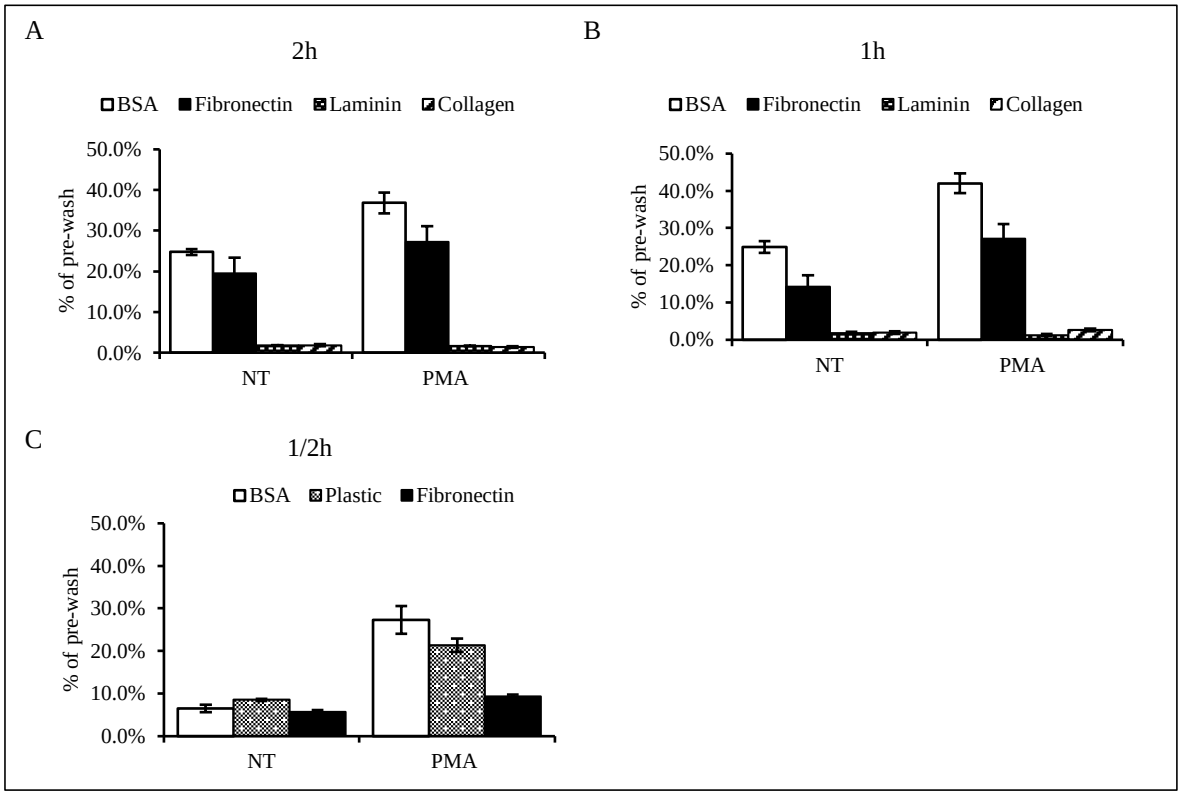

Figure 17. Effect of PMA on BV-2 adhesion after 3 washes. BV-2 cells were in HBSS + 1% BSA medium, labelled with fluorescent dye Calcein, washed 1x with medium, and activated by PMA for 20min before measurements. NT = no treatment, PMA = 50ng/mL. Values are presented as mean ± SEM. n=3. Statistical significance compared to the PMA group is marked. (* p-value <0.05). A) Cells were activated for 2h in PMA. 50µL of 3% BSA, fibronectin, laminin, and collagen were used as adhesion substrates. B) Cells were activated for 1h in PMA. 50µL of 3% BSA, fibronectin, laminin, and collagen were used as adhesion substrates. C) Cells were activated for 1/2h in PMA. 50µL of 3% BSA, fibronectin, laminin, and collagen were used as adhesion substrates. Direct adhesion to the plate was measured as well.

In addition, BV-2 cells showed activation-time-dependent adhesion. Stimulated BV-2 cells adhered better to 3% BSA as activation time in PMA shortened from 2h to 1/2h (Figure 18).

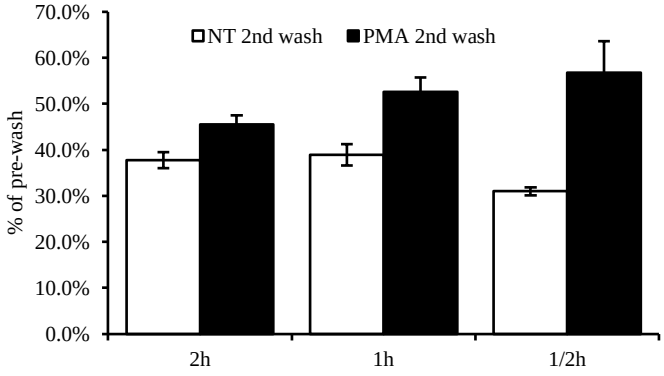

Figure 18. Effect of PMA on BV-2 adhesion onto 3% BSA after 2 washes. BV-2 cells were in HBSS + 1% BSA medium, labelled with fluorescent dye Calcein, washed 1x with medium, and activated by PMA for 20min before measurements. NT = no treatment, PMA = 50ng/mL. Values are presented as mean ± SEM, n=3.

Discussion

Previous research (Yang et al., 2016) suggested that serpins could reduce the activation of microglial cells and thus be inhibitory on neuroinflammation – an important pathological factor in several neurodegenerative diseases (Cherry et al., 2014). The aim of my thesis was to assess the effect of tick protease inhibitors – serpins and cystatins – on the microglial cell line BV-2. Their inhibitory function on neuroinflammation could have a therapeutic potential.

Some of the tested tick proteins have been characterized previously, while the function of others is still unknown. *Ixodes ricinus* serpin IRS-2 has been reported to inhibit inflammation (Chmelar et al., 2011) and reduce IL-6 production (Palenikova et al., 2015); unpublished lab data suggested that cystatin G1 could be anti-inflammatory as well; sialostatins L1 and L2 – cystatins from *Ixodes scapularis* showcased the ability to lower pro-inflammatory cytokine production in dendritic cells (Chen et al., 2014; Horka et al., 2012; Lieskovska et al., 2015). However, these protease inhibitors have not yet been tested on microglial cells. Therefore, cystatins G1, G2, G9, G16, L1, and L2, and serpins S8K and IRS-2, were assessed for their ability to suppress the release of pro-inflammatory mediators such as nitric oxide, TNF-α, and IL-6 in LPS-activated BV-2 mouse microglial cells.

Prior to the testing of inhibitors, various cell growth conditions were tested to find an optimal configuration. The ideal condition for a timely cell growth was found to be 80,000 cells/well in a 24-well plate incubated for 24h.

Some studies have used LPS and IFN-γ combined (Miyake et al., 2014; Okorji & Olajide, 2014; Park et al., 2014) as the inflammation stimulation system while others used LPS (Cheong et al., 2011; Dong et al., 2014; Gold et al., 2014) or IFN-γ (Zhou et al., 2015) alone. All three options were applied, namely LPS at 100ng/mL and IFN-γ at 100U/mL. Nitric oxide (NO) production is a common marker used to evaluate inflammation in BV-2 cells (Cheong et al., 2011; Dong et al., 2014; Gold et al., 2014). As expected, all three methods successfully stimulated BV-2 cells – nitric oxide levels in stimulated groups were significantly higher than the baseline production (Figure 8A). Contrary to the documented synergic activation by LPS and IFN-γ combined (Schroder et al., 2004), LPS+IFN-γ did not simulate BV-2 cells any better than LPS did alone. Another study experienced a similar contradiction in cytokine levels – treatment with LPS+IFN-γ resulted in lower levels of

TNF-α and IL-6 than that when exposed to only LPS (Papageorgiou et al., 2016). In agreement with previous research (Cheong et al., 2011; Dong et al., 2014; Gold et al., 2014; Zhou et al., 2015), my data suggests that stimulation using LPS, IFN-γ, or LPS+IFN-γ was competent in inducing inflammation in BV-2 cells, although the documented synergic action of LPS+IFN-γ (Schroder et al., 2004) was not observed.

Dexamethasone (DMZ), a synthetic glucocorticoid prescribed for treating various inflammatory conditions such as multiple sclerosis, head injury, and allergies, (Drugs.com, 2016), was tested at different concentrations in LPS-stimulated BV-2 cells. Dexamethasone was reported to inhibit NO production in LPS-stimulated J774 macrophages, in a dose-dependent manner from 0.1μM (39ng/mL) to 1 μM (Korhonen et al., 2002). Here, only at 500ng/mL, DMZ showed significant inhibition on NO production (Figure 8B). The calibration curve made from standard solutions could not sustain linearity after a certain concentration level, possibly due to insufficient reaction between the nitrite and the sulphanilic acid of the old Griess reagent, resulting in a shortened working range. A fresh Griess reagent was used in the next experiment (Figure 8C) and the calibration curved behaved as it should. Experimental error as well as the defect Griess reagent could have been the reason for deviant results.

In a preliminary screening of the protease inhibitors, LPS+IFN-γ significantly increased NO production as expected, but among the inhibitors only G1 showed a slight reducing effect while the others had virtually no influence (Figure 8C). Without precise information on the tested cystatins, it would be difficult to justify the results, not to mention that the function of cystatins is not universally agreed upon: human cystatin C has been found to enhance NO production and TNF-α in mouse peritoneal macrophages (Frendeus et al., 2009) while down-regulating TNF- α and IL-1β in human monocytes (Gren et al., 2016). Chicken cystatin has also been reported to enhance NO production in mouse peritoneal macrophages (Verdot et al., 1999). L1 and L2 have demonstrated abilities to decrease pro-inflammatory cytokines in dendritic cells (Horka et al., 2012; Sa-Nunes et al., 2009), but the anti-inflammatory effect was not apparent in NO production in BV-2 cells. Cystatin C and sialostatins L1 and L2 are all potent inhibitors of cathepsins L and S (Kopitar et al., 2006, Kotsyfakis et al., 2010), yet their function on inflammation seems to diverge. In addition to the uncertainty of cystatin function, the protocol to measure NO production in BV-2 cells was adopted from one for PMJ2R and IC-21 macrophage cells, which may require further optimization to produce

accurate results in BV-2 cells. The experimental data does not support the hypothesized suppressive effect of the tested protease inhibitors on NO production.

Cytokines TNF-α and IL-6 are two major mediators and indicators of inflammation. Many studies have reported on their increase upon stimulation of BV-2 cells and decrease when inhibitors are used (Cheong et al., 2011; Dong et al., 2014; Gold et al., 2014). While LPS at 100ng/mL successfully enhanced production of both of the cytokines (Figure 9-11), the inhibitors showed perplexing effects.

The level of IL-6 was enhanced by G1 and G2 (Figure 10, 12A), contrary to the anti-inflammatory nature they were predicted to possess. As in NO production, the effect of cystatins on pro-inflammatory cytokines varies from study to study: human cystatin C enhanced NO production and TNF-α in mouse peritoneal macrophages (Frendeus et al., 2009) but decreased TNF- α and IL-1β in human monocytes (Gren et al., 2016). G1 and G2 could behave similarly to those cystatins which enhance TNF-α in mouse peritoneal macrophages (Frendeus et al., 2009). Once structural information on the tested cystatins becomes available, protein function prediction could help narrow down the explanation, along with more experimental testing.

The inhibitors had similar effects on TNF-α production (Figure 11A-C) as on IL-6 production. Figure 11D represents results that are rather different from the other experiments – although all tested inhibitors showed strong anti-inflammatory effect, the result could not be reproduced. Upon closer inspection, its TNF-α concentration in LPS group (Figure 11D) was much higher than that from the other experiments (Figure 11 A-C) – 10ng/mL compared to 2-4 ng/mL, respectively. This result likely was due to experimental error therefore was excluded from the normalization calculations. The enhancing effect of G1 on TNF-α and IL-6 production, despite in varying intensity, was consistent and replicable (Figure 10, Figure 11). Other tested proteins continued to show no influence (Figure 12). A similar experiment was conducted on IC-21 cells, a mouse peritoneal macrophage cell line, to compare the responses between microglia and their close relative macrophage. Yet again, G1 demonstrated a pro-inflammatory effect (Figure 14A).

Contrary to its predicted function as a tick salivary cystatin, G1 repeatedly increased cytokine levels in stimulated BV-2 cells. Another cystatin, CysC, has been reported to

increase TNF-α level in mouse peritoneal macrophages (Frendeus et al., 2009). A plausible explanation would require further experimentation and information on the structure of G1.

Adhesion assays were used to assess the activation state of microglia since the ability to migrate and attach to surfaces is crucial to their immune functions (Milner & Campbell, 2002). Stimulation with PMA yielded perplexing results: in some experiments PMA reduced (Figure 16) or had no effect (Figure 17) on BV-2 cells adhesion. This contradicted with the report that macrophage adhesion to laminin can be triggered by protein kinase C activators such as PMA (Mercurio & Shaw, 1988) and the adhesion-enhancing effect of PMA observed in another study (Milner & Campbell, 2002). Another set of experiments, however, was in agreement with a study by Milner in 2002 – stimulation with PMA significantly increased microglia adhesion to laminin and fibronectin, with adhesion to fibronectin much stronger than to laminin and collagen (Figure 18A, B). The weak attachment to laminin is not only present in microglia, but also in neutrophils and macrophages in resting state (Milner & Campbell, 2002). The same research group also reported that microglia attach strongly to cell culture plate plastic, as observed in this experiment (Figure 18C). The adhesion-enhancing effect of G1 (Figure 15, 16) is in accordance with the experimental data that G1 increased TNF-α production (Figure 12B) and with a study which reported increased adhesion of microglia to laminin after administration of TNF-α and IFN-γ (Milner & Campbell, 2002).

However, these results cannot be considered reliable due to the unsuccessful stimulation by PMA on cell adhesion. One confounding factor contributing to the inconsistent results of adhesion assays may be Calcein, the fluorescent dye used to label BV-2 cells. It has been reported that Calcein fluorescence may be increased or decreased by treatment conditions due to alteration in intracellular conversion (Miles et al., 2015).

In summary, the experimental data suffered from large variance; optimization and fine-tuning of protocols to generate more consistent and uniform results would be necessary to enhance reproducibility. Nevertheless, the pro-inflammatory behavior of G1 on BV-2 cells was rather consistent. Although surprising, it might not be wrong. The traditional role of cystatins as inhibitors of cathepsins – proteases involved in antigen presentation (Kopitar-Jerala, 2006) – suggests an anti-inflammatory function while recent studies have introduced their pro-inflammatory properties (Frendeus et al., 2009; Kopitar-Jerala, 2006; Verdot et al.,

1999). The unexpected response of G1 and other protease inhibitors tested in this project may be part of the complexity of their functions.

Conclusion

With knowledge derived from previous studies, it was hypothesized that cystatins G1, G2, G9 and G16, serpins IRS2 and S8K, and sialostatins L1 and L2, might possess anti-inflammatory properties. This project aimed to evaluate the aforementioned protease inhibitors on their effect on activated microglia, as part of the effort to improve our understanding and advance the development of potential treatments of neurodegenerative diseases.

During my work, the optimal cell growth condition was investigated and implemented in later experiments; different methods to stimulate microglia inflammation using LPS and/or IFN-γ were tested and found to be efficient in increasing production of nitric oxide, TNF-α, and IL-6 by BV-2 cells.

Much to the contrary of the hypothesis, most protease inhibitors had no effect on inflammation in microglia – G9, G16, IRS2, S8K, L1 and L2 did not influence the production of nitric oxide, TNF-α, or IL-6 in any statistically significant way. Instead of the assumed anti-inflammatory function of cystatins G1 and G2, the data suggests that they are pro-inflammatory – G1 significantly increased cytokine levels of TNF-α and IL-6, and G2 enhanced IL-6 production.

Given the complex, sometimes opposite, roles of cystatins on inflammation, the observed pro-inflammatory effect of cystatins G1 and G2 may be appropriate. Further investigations may be interesting and of scientific value.

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