T.C.
DOKUZ EYLÜL UNIVERSITY
IZMIR INTERNATIONAL BIOMEDICINE AND GENOME INSTITUTE

INVESTIGATION OF THE EFFECTS OF DIETHYL MALEATE ON NLRP3 INFLAMMASOME ACTIVATION IN MICROGLIAL CELLS

ÇAĞLA KİSER

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

MASTER OF SCIENCE THESIS

İZMİR – 2020

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SUPERVISOR: PROF. DR. ŞERMİN GENÇ

Dokuz Eylül Üniversitesi İzmir Uluslararası Biyotıp ve Genom Enstitüsü Genom Bilimleri ve Moleküler Biyoteknoloji Anabilim Dalı,
Moleküler Biyoloji ve Genetik Yüksek Lisans programı öğrencisi Çağla Kiser
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Dokuz Eylül University Izmir International Biomedicine and Genome Enstitute Department of Genomics and Molecular Biotechnology, Molecular Biology and Genetics graduate program Master of Science student Çağla Kiser has successfully completed her Master of Science thesis titled **'INVESTIGATION OF THE EFFECTS OF DIETHYL MALEATE ON NLRP3 INFLAMMASOME ACTIVATION IN MICROGLIAL CELLS'** on the date of 13 / 01 / 2020.

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LIST OF ABBREVIATIONS

AD: Alzheimer's Disease

ATP: Adenosine triphosphate

cDNA: Complementary DNA

CNS: Central Nervous System

CSF-1: Colony stimulating factor-1

DAMP: Damage-associated Molecular Pattern

FBS: Fetal Bovine Serum

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

GSDMD: Gasdermin D

HRP: Horseradish Peroxidase

IFN: Interferon

IL: Interleukin

IRF-8: Interferon regulatory factor-8

Keap1: Kelch-like ECH-associated protein-1

LDH: Lactate Dehydrogenase

LPS: Lipopolysaccharide

mRNA: Messenger RNA

MS: Multiple Sclerosis

NF-κB: Nuclear Factor kappa-B

NLRP3: NOD-like receptor family, pyrin domain containing 3

NLRs: NOD-like receptors

Nrf2: Nuclear factor-erythroid-2-related factor 2

PAMP: Pathogen-associated Molecular Pattern

PD: Parkinson’s Disease

PI: Propidium Iodide

PRR: Pathogen Recognition Receptor

qPCR: Quantitative PCR

ROS: Reactive oxygen species

TLR: Toll-like receptor

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INVESTIGATION OF THE EFFECTS OF DIETHYL MALEATE ON NLRP3 INFLAMMASOME ACTIVATION IN MICROGLIAL CELLS

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ABSTRACT

Microglial cells are tissue-resident macrophages of the Central Nervous System (CNS) (Dubbelaar, Kracht et al. 2018). In case of an injury in the brain, microglia act through their phagocytic function and eliminate invading microbes and other substances that harm the CNS. Also, they are responsible for secretion of cytokines assisting the immune response within the CNS (Dubbelaar, Kracht et al. 2018).

Inflammasome activation initiates inflammatory responses leading to secretion of IL-1β and IL-18 cytokines and activation of caspase-1, and may also lead to pyroptotic cell death (Yang, Wang et al. 2019). For this reason, inflammasome activation should be prevented under such conditions. Chemicals with anti-inflammatory effects are appropriate candidates to inhibit inflammasome activation. Diethyl Maleate (DEM) is a Nrf2 activator and correlatively, DEM has been estimated to inhibit secretion of inflammatory cytokines and possible cell death. It is hypothesized that DEM represses LPS+ATP induced NLRP3 inflammasome activation caused cell death of N9 cells.

In this thesis, effects of DEM on NLRP3 inflammasome activation in N9 microglia was investigated. NLRP3 inflammasome activation via LPS+ATP was performed. N9 cells were treated via DEM in order to determine the effect on inflammasome activation. The cytotoxicity of DEM in regarding conditions was determined via LDH (Lactate Dehydrogenase) and PI visualization. Afterwards, inflammasome markers were examined at mRNA level via PCR, at protein level via Western Blotting and ELISA. It was determined that DEM pretreatment represses inflammatory cytokine secretion and cell death arising from NLRP3 inflammasome activation.

Keywords: Diethyl Maleate, microglia, N9, inflammasome, NLRP3, pyroptosis, LPS, ATP

MİKROGLİAL HÜCRELERDE DİETİL MALEAT'IN NLRP3 İNFLAMAZOM AKTİVASYONU ÜZERİNE ETKİLERİNİN ARAŞTIRILMASI

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ÖZET

Mikroglial hücreler merkezi sinir sisteminin (MSS) dokularda yaşayan makrofajlarıdır (Dubbelaar, Kracht et al. 2018). Beyinde bir akut hasar oluştuğunda, mikroglia, hücresel kalıntıları, invaze eden mikrobiyel organizmaları ve MSS'ye zararı olan diğer bileşenleri fagosite ederler. Diğer bir yandan, sitokinleri salgılamakla sorumludurlar. Bunu merkezi sinir sisteminde immün yanıta destek olarak gerçekleştirirler (Dubbelaar, Kracht et al. 2018).

İnflamazom aktivasyonu, IL-1β ve IL-18 sitokinlerinin salınımına, Kaspaz-1 aktivasyonuna ve olası piroptotik hücre ölümüne yol açan inflamatuar yanıtı başlatır (Yang, Wang et al. 2019). Bu sepeble, bu gibi durumlarda inflamazom aktivasyonunun önlenmesi gerekmektedir. Anti-inflamatuar özellik gösteren birtakım kimyasallar inflamazom aktivasyonunu engellemek adına uygun adaylar olabilmektedir. Dietil Maleat bir Nrf2 indükleyicisidir ve Nrf2 yolağına bağlı olarak, DEM'in, inflamatuar sitokinlerinin salınımını ve olası hücre ölümünü inhibe edeceği öngörülmektedir. DEM'in LPS+ATP ile indüklenmiş NLRP3 inflamazom aktivasyonu kaynaklı hücre ölümünü baskıladığı hipotez edilmektedir.

Bu tezde, DEM'in N9 mikroglial hücre hattında NLRP3 inflamazom aktivasyonu üzerindeki etkisi incelenmiştir. Bu amaçla, N9 hücre hattında NLRP3 inflamazom aktivasyonu LPS ve ATP ile indüklenmiştir. Hücreler, inflamazom aktivasyonu üzerindeki etkisini belirlemek adına DEM ile muamele edilmiştir. DEM'in uygulanan koşullar altında sitotoksisitesi LDH (Lactate Dehydrogenase) ve PI görüntüleme yöntemleriyle belirlenmiştir. Sonrasında, inflamazom markörleri mRNA düzeyinde PCR ile, protein düzeyinde ise Western Blotlama ve ELISA yöntemleriyle incelenmiştir. DEM ön muamelesinin NLRP3 inflamazom aktivasyonu kaynaklı inflamatuar sitokin salınımını ve hücre ölümünü baskıladığı belirlenmiştir.

Anahtar kelimeler: Dietil Maleat, mikroglia, N9, inflamazom, NLRP3, piroptoz, LPS, ATP

1. INTRODUCTION AND AIM

1.1. Statement and Importance of the Problem

NLRP3 inflammasome is activated upon sensing PAMPs (pathogen-associated molecular pattern) and DAMPs (damage-associated molecular pattern). Following the activation, pro-inflammatory cytokines are secreted to immediate environment, leading to activation of nearby microglial cell population; also, inflammasome activation is capable of resulting in Caspase-1 dependent cell death called pyroptosis (Guo, Callaway et al. 2015). Imbalance in microglial activation results in excess inflammation and contributes to neurodegenerative diseases including Alzheimer's (AD), Parkinson's diseases (PD), dementia and Multiple Sclerosis (MS) (Colonna and Butovsky 2017). DEM as a repressive agent, is a potential agent to repress excess inflammation of the activated microglia, contributing to reduction of the progressive effects of aforementioned conditions and diseases.

1.2. Aim of the Study

The goal of the study is investigation of the effects of Diethyl Maleate on NLRP3 inflammasome activation in microglial cells via using DEM as a repressive agent in order to prevent excess inflammation resulting from imbalanced microglia function. Aim of the study is to determine the effects of DEM on inflammasome activity and regarding changes of inflammasome parameters IL-1β, IL-18, Caspase-1 and NLRP3 in microglial cells; and to determine effects of DEM on NLRP3 inflammasome-induced cell death of N9 murine microglial cell.

1.3. Hypothesis of the Study

DEM represses NLRP3 inflammasome activation in LPS + ATP induced cell death of N9 murine microglial cells.

2. GENERAL INFORMATION

2.1. Microglial Cells

Microglial cells are the corresponding professional phagocytic, tissue-resident macrophages (Dubbelaar, Kracht et al. (2018) and primary effector immunity of the CNS (Prinz, Jung et al. 2019). Microglial cells comprise approximately 10 % of the cells within the central nervous system and they dominate within the network (Colonna and Butovsky 2017). Throughout the CNS consisting the brain and the spinal cord, microglial cell populations appear to be uniformly distributed (Ginhoux and Prinz 2015). This population of cells belong to glia family that are non-neuronal cells, and they are responsible for support as other glial cell populations (Ginhoux and Prinz 2015). Microglial cells are induced via signals from PAMPs or DAMPs through similar pathways (Kettenmann, Hanisch et al. 2011) and they are known to move or extend through their protrusions immediately to damaged domains (Kettenmann and Verkhratsky 2008). Microglia contributes to CNS homeostasis and also inflammatory mechanisms through their characteristics including antigen presentation and cytokine secretion (Wolf, Boddeke et al. 2017). Dysfunction in microglia and imbalanced homeostasis could lead to such neurodegenerative disorders with severe onset (Tay, Savage et al. 2017).

2.2. Microglial History and Origin

Tissue macrophages collectively have been thought to be from identical origin in the past. It was only known that progenitors of microglia invade brain in early embryonic development. Microglia colonization in early embryonic brain is a common process among vertebrate species, which signifies importance and necessity of microglia in early brain development (Ginhoux and Prinz 2015). In initial studies regarding microglial cells, these cells were observed to be present in early development which lead to the assumption that microglia arise from embryonic progenitors. Rio-Hortega suggested these progenitors were meningeal macrophages infiltrating within the brain in early embryonic development. However, this was a dilemma at the time. Rio-Hortega and also members of scientific community then, thought that microglial cells could be originated from blood monocytes (Ginhoux and Prinz 2015). Following observations of microglial cells at embryonic day 9.5 of murine brain development have driven researchers to further investigate this complex process of microglia origination. Observing microglia at E9.5 lead to the idea that microglia precursors arise from yolk sac (YS). This YS origin of microglia

provides an incomparable aspect among tissue macrophages. Also, the studies demonstrated that embryonic hematopoietic precursors of microglia locate within the CNS before birth (Ginhoux and Prinz 2015).

Rudolf Virchow conceptualized glia in 1856 as a member of brain cell population other than the neurons (Wolf, Boddeke et al. 2017). Later on, in early 20th century, Ramon y Cajal described a third concept besides the neurons and astrocytes. Meanwhile, a definite cell type was recognized to be involved in several disease states including dementia, MS (Multiple Sclerosis), Alzheimer's disease and brain injury (Wolf, Boddeke et al. 2017). The notion of microglia was made widely known by Pio del Rio-Hortega as a declared member of CNS (Kettenmann, Hanisch et al. 2011). In 1919, Rio-Hortega published four papers highlighting the concept of glia and discriminating the astrocyte, microglia and oligodendrocyte concepts, which are valid today (Wolf, Boddeke et al. 2017). Thereby, the first definite classification of microglia as distinct cellular elements of CNS (Kettenmann, Hanisch et al. 2011), phagocytic, migrating population of cells within the CNS was established by Rio-Hortega. Rio-Hortega named these cells as microgliocytes (Wolf, Boddeke et al. 2017). He stated microglial cells to be from mesodermal origin. Later, Rio-Hortega has brought up the term microglial cell and he has added to description regarding these cells as non-neuronal components belonging to the CNS, and also are distinguishable from oligodendrocytes (Ginhoux and Prinz 2015). Pio del Rio-Hortega proposed a number of definitions regarding microglia some of which are that microglial cells possess amoeboid morphology they are of mesodermal origin; they migrate through vessels and white matter; they have a ramified resting state with their processes; they have their own territories where they are located and act. These definitions are still valid today (Kettenmann, Hanisch et al. 2011).

Several other researches in the field have being held, importantly, pathologists had an attention to a definite cell type in the brain of a person with neurodegenerative disease. Alois Alzheimer and Franz Nissl defined those cells as granular cells along with other various descriptions including rod cells and clearance. Consequently, it was determined that these cell types are residing in numerous types of brain diseases and brain injury (Wolf, Boddeke et al. 2017).

2.3. Microglia in Different Species

Up to the present, most of the research with regard to microglia has been done via rodent and mouse models and comparatively, limited research has been conducted to investigate the resemblance of microglia functions in humans. A core set of microglial genes has been determined to be conserved among mice as well as humans. On the other hand, only 30% of human microglia genes are known to be leveraged in microglia of mouse. Regarding numerous differences between mouse and human, phagocytosis is regulated by molecules such as C4a, C4b and Siglec-11 are appear to be present in humans but not in mouse models; similarly, Siglec-H and Fcrls molecules which are involved in phagocytosis are present in mice but not in human. Despite these differences, several essential similarities between human and mice microglia render continuum of use of rodent models in developmental microglia researches. As an example, colonization of microglia within human brain takes resembling period of time to rodent microglia colonization (Lenz and Nelson 2018).

2.4. Microglia Development

Rio Hortega stated that microglia, of mesodermal origin, enter the brain early in development (Wolf, Boddeke et al. 2017). However, microglia is now known to develop in early embryogenesis along with a pool of primitive macrophages from the precursor cells of myeloid lineage within the embryonic yolk sac and around the embryonic day 8,5 (mouse) they start to migrate (Kabba, Xu et al. 2018). Precursors of microglia appears at the base of fourth ventricle at nearly embryonic day 13. In humans, at the 13^{th} week of gestation, cell populations alike microglia can be observed, however, ramified, differentiated microglia appear later at 21^{th} week (Wolf, Boddeke et al. 2017).

CSF-1 (Colony Stimulating Factor 1) signaling is a significant aspect for microglia development. A mouse model of CSF-1 receptor deficiency demonstrated that microglia is significantly decreased in number along with some other macrophages within the tissue (Wolf, Boddeke et al. 2017). CSF-1 and/or IL34 is ligands for this receptor and DAP12 is an essential adaptor protein for CSF-1 receptor for the process of microglia development. Likewise, IRF-8 (interferon regulatory factor-8) seems to be important for the microglia development as it has been observed in its deficiency microglial concentration has decreased (Wolf, Boddeke et al. 2017).

2.5. Homeostatic Microglia

Microglia is one of the slowest dividing immune cells relative to other immune cells which are tend to live no longer than a couple of days or weeks. In comparison, turnover rate of human microglia is profoundly lower than rate in mice microglia. On the other hand, mice microglia have a higher exchange rate regarding myelin and cell debris clearance (Reu, Khosravi et al. 2017). In regard to self-renewal ability of microglia, it is supposed that the adult microglial population is long lived and maintained by coupled actions proliferation and apoptosis mechanisms and their fine balance. This coupling allows steadiness of microglial cell numbers from postnatal to aging time periods both in mice and human. The mentioned long-lived terminology refers to population maintenance but not individual cells, for sure, cells are going through apoptosis when they complete their lifetime. In a research conducted by Askew et.al. (2017), it was determined that the murine population of microglial cells was significantly maintained with slight changes throughout a lifetime, from youth (4-6 months) to aging (24 months) brains in all investigated parts of the brain. Exceptionally, thalamus did not follow the trend and demonstrated a rise in number of cells with aging in the study. The same study indicates that in human microglia in temporal cortex, there is no significant change in microglial cell density from young (20-35 years old) to aged (58-76 years old). Microglia composition in murine brain is found to be enriched in grey-matter regions, conversely, white matter is denser in human. This observation suggests that regional differences arise in microglial population as species-specific manner (Askew, Li et al. 2017). To compare the brain regions in terms of microglia populations; in hippocampus, olfactory bulb and cerebellum turnover is more rapid than turnover in the cortex, midbrain and hypothalamus (Sominsky, De Luca et al. 2018).

To evaluate mice and human microglia together, overall, microglia turnover rate is significantly fast with high proliferation rate, enabling the population to be renewed recurrently within a lifetime. The microglia composition is replaced within a couple of weeks along with death of cells, self-renewal and changes in positions of cells. Microglial cell density is maintained with aging in both mice and human. However, the exact dynamics of the above-stated population is not clear. To summarize, microglia is considered to be continuously and rapidly remodeled population of cells which justifies its importance in insurance of brain homeostasis but no precise knowledge about temporal and spatial dynamics of microglia has been conducted yet (Askew, Li et al. 2017).

2.6. Microglia Function

Microglial cells appear to be present throughout each region in healthy CNS and play role in numerous different functions during the development of CNS where they coordinate it in forming the network (Kierdorf and Prinz 2017). The principal role of this innate immune system is the rapid identification and response to the pathogenic elements entering within the body, contributing to the brain homeostasis (Colonna and Butovsky 2017). In prenatal brain, the wiring of circuits of forebrain is regulated by microglia through regulation of dopaminergic axons and emplacement of interneurons (Ginhoux and Prinz 2015). Microglia, has several functions including, remarkably, contributing the homeostasis of the brain, phagocytosis of the dead cells, supporting the neurons in the CNS during development, organizing the dynamics of synapse formation, being involved in development of vasculature and neuronal circuits (Kierdorf and Prinz 2017). In addition to expressing receptors including ones for neurotransmitters, microglial cells are able to communicate with macroglia, neurons and immune cells via variety of signaling pathways (Kettenmann, Hanisch et al. 2011). Nonetheless, microglia are important contributor and regulator of synaptogenesis, survival of synapses or pruning of them, maturation of neurons and their activity. Microglia is responsible for the programmed cell death which is important for the clarification of the neuronal network. Microglial cells phagocyte the apoptotic cells and damaged agents through secreting cytokines in order to lead the process and producing reactive oxygen species. The knowledge of role of microglia in response to injury to the CNS is well established. In case of an acute injury, microglial cells act through phagocytosis of the cellular debris, which is a process known to be mediated by a number of receptors on the cell surface responding to the signals from the microenvironment (Sominsky, De Luca et al. 2018). Microglia is capable of producing various signaling molecules that can be cytokines, neurotransmitters and extracellular matrix proteins that are involved in regulation of synaptic activity and plasticity. Dysfunction of microglia could result in interference of neuronal activity and impaired clarification of debris. This dysfunction in microglia and imbalanced homeostasis could lead to neurodegenerative disorders with severe onset (Tay, Savage et al. 2017). In addition, microglia play role in prevention of excitotoxicity. Excitotoxicity results from excess release of neurotransmitters leading to long-time depolarization state of neurons, neurotoxicity and swelling of axons. Signals from the damaged and dysfunctional neurons activate the microglia and along with being induced via these signals, microglial cells secrete several cytokines in order to temper the

toxicity. Also, processes of the microglia temper the axon swelling via wrapping around them and induces the repolarization state all together preventing the excitotoxicity (Colonna and Butovsky 2017).

2.7. Microglia Activation

All over the CNS, in homeostatic conditions within a healthy brain, microglial cells are observed in a ramified morphology along with a small soma and motile branches (also termed protrusions or processes), this state is also known as resting phenotype, that microglial cells actively scan their territory of microenvironment against any harm and danger signals. The process of identification and elimination through inflammatory activation process is conjugated via receptor-ligand interactions of several membrane-bound as well as cytosolic receptors (Colonna and Butovsky 2017). Microglial cells have been determined to express variety of genes related to myeloid lineage, including PAMP (pathogen-associated molecular pattern) and DAMP (damage-associated molecular pattern) receptors. These receptors are all together named as pathogen recognition receptors (PRRs). PRRs are comprised of Toll-like receptors (TLRs), NOD-like receptors (NLRs), nucleic acid receptors, C-type lectin receptors and their coreceptors. To add more, numerous receptor families for phagocytosis and endocytosis of cells going through apoptosis, aggregates of proteins and particles of lipoproteins (Colonna and Butovsky 2017). In addition, expression of phagocytosis and antigen presenting genes was also confirmed, which contributes to brain homeostasis (Dubbelaar, Kracht et al. 2018). When a damage to brain or infection is detected, microglia are activated and following this activation, numerous inflammatory cytokines, mediators and neurotoxic factors are known to be secreted by microglia (Choi, Koppula et al. 2011). By the time microglial cells detect signs for lesions or any dysfunction involving infection, trauma, neurodegenerative diseases or loss of brain homeostasis, or. cells going through programmed cell death, they transform into activated state and migrate to the related site (Wolf, Boddeke et al. 2017). Microglia then trigger and act to maintain the inflammatory response to regarding damage, infection or dysfunction (Choi, Koppula et al. 2011). Regarding their phagocytic functions, they tend to eliminate either whole cells or their sub-structures, importantly synapses (Wolf, Boddeke et al. 2017). Activated state of microglial cells are related to response to varying stimuli which they recognize through activated pathways of their related PRRs (pathogen recognition receptors) (Dubbelaar, Kracht et al. 2018).

In phenotype, during microglial activation, cellular processes of microglial cells are decreased in number, they acquire a thicker configuration, and cells gain amoeba-like appearance. Along with these changes in their protrusions, microglial cells enlarge the size of their cell bodies. They also increase via proliferation upon activation. Additionally, an important characteristic of microglia is that they have a low activation threshold and relevant activation is very fast, happening within a number of ten minutes. This competency of rapid respond is crucial in case of trauma, ischemia and so on. Considering all together, activation of microglia is a highly regulated process (Kabba, Xu et al. 2018).

2.8. Inflammasome

Inflammasomes are multi-protein complexes that are responsible for mediating host immune responses (Song and Li 2018). Inflammasomes are responsible for triggering caspase activation and are ultimately involved in proteolytic cleavage of pro-IL-1β and pro-IL-18 cytokines and following release of these cytokines (Mamik and Power 2017). The basic composition of a canonical inflammasome is a sensor comprised of a pattern recognition receptor (PRR), an adaptor protein (ASC) and an effector protein as pro-caspase-1 (Song and Li 2018). The sensor proteins of these complexes are cytosolic proteins with ability to recognize PAMPs and/or DAMPs (Man and Kanneganti 2015). Inflammasome with varying sensor receptors are similar in their downstream mechanisms, however, each responds distinct type of stimuli. Until now, inflammasomes are categorized in five units regarding their core pattern recognition receptors (PRRs): NLRP1, NLRP3, NLRC4, Pyrin, AIM2 (Absent in Melanoma 2) (Song and Li 2018).

Amongst, NLRP3 inflammasome is well-characterized and it is essential due to modulating the maturation of two important proinflammatory cytokines from interleukin-1 family which are IL-1β and IL-18 (Jo, Kim et al. 2015). The general function of inflammasomes is to lead autocatalysis and activation of pro-caspase-1, following the maturation of caspase-1, precursors of IL-1β and IL-18 pro-inflammatory cytokines are cleaved into their mature forms. Additionally, pyroptosis is induced (He, Hara et al. 2016). Inflammasome machinery is not only present in a distinct type of cells but is determined to be involved in several cell types where it contributes to activation of innate immunity in numerous organs and parts of the body, including the central nervous system, too (Mamik and Power 2017). Pattern recognition

receptors are activated by PAMPs or DAMPs, leading to downstream trigger of signaling cascades (Guo, Callaway et al. 2015).

2.8.1. NLRP3 inflammasome

NLRP3 inflammasome has been widely investigated and intensely studied unlike other inflammasomes, due to the fact that NLRP3 inflammasome is involved in numerous types of diseases (He, Hara et al. 2016). NLR protein family includes NLRP3 where its 22 members are present in humans and 34 members are known to be present in mouse (He, Hara et al. 2016). NLRP3 was discovered to be involved in a broad spectrum of disease models. Therefore, understanding NLRP3 inflammasome machinery and revealing the intrinsic regulation of the machinery is considerably important in potential NLRP3-targeted therapies (Swanson, Deng et al. 2019).

NLRP3 is NOD-like receptor with pyrine domain protein-3 and is a cytosolic receptor sensing a wide range of endogenous danger signals and microbial agents (Swanson, Deng et al. 2019). NLRP3 involves a pyrine domain at amino terminal, a NACHT domain at the center and a leucine rich domain at the carboxy-terminal (Swanson, Deng et al. 2019).

An important aspect of NLRP3 inflammasome is that, in addition to responding to danger signals from microbial agents or microorganisms itself, the regarding inflammasome is also response to signals of metabolic dysregulations (Tschopp and Schroder 2010).

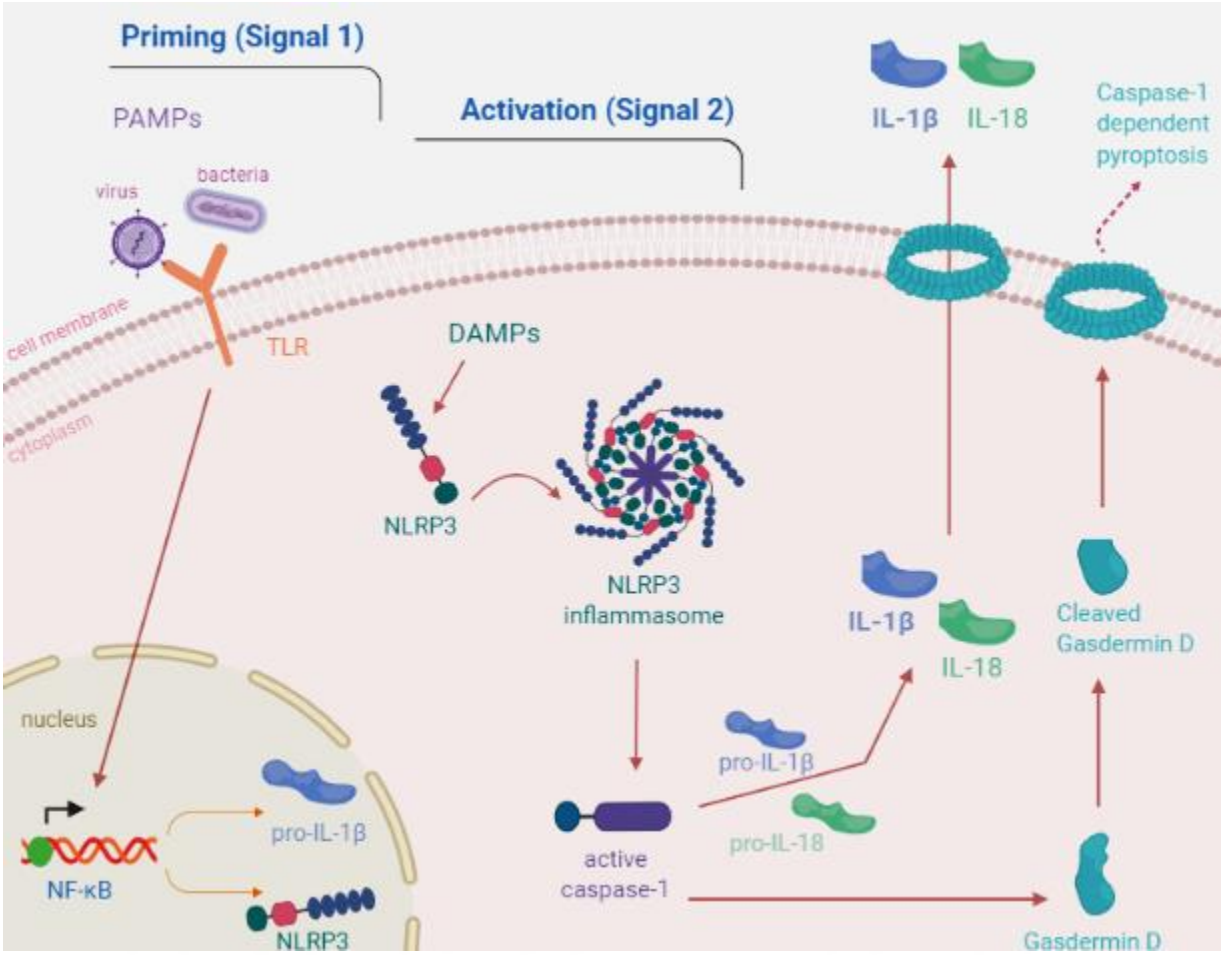
2.8.2. Inflammasome activation

The primary role of the innate immune system is to provide homeostasis. In case of an interference to homeostasis such as cellular stress, damages or infection PRRs sense these threats and act to response (Man and Kanneganti 2015). NLRP3 is distinct as it is activated through a variety of stimuli that could be bacterial, viral, fungal infections, and also through sterile inflammation by endogenous DAMPs (Swanson, Deng et al. 2019). Stimuli are PAMPs or DAMPs such as numerous bacterial toxins, bacterial mRNA, bacterial hybrids of DNA:RNA, muramyl dipeptide, virus DNA or RNAs, ATP, uric acid crystals, silica and asbestos (Man and Kanneganti 2015). It has been later on determined that most of the stimuli from bacterial origin, including TLR ligands and muramyl dipeptide do not act to activate NLRP3 inflammasome

directly, rather, they act to prime inflammasome activation (He, Hara et al. 2016). Moreover, ATP or pore forming toxins are capable of inducing NLRP3 activation. Hence, NLRP3 inflammasome activation was suggested to possess a two-signal model: first signal referred as 'priming' and the second signal referred as 'activation' (He, Hara et al. 2016). TLRs, NOD2, TNFR1 or TNFR2 provides the signal for priming, where NF- κB-regulated expression of NLRP3 increases (Man and Kanneganti 2015). NF- κB activation is important in order for increasing expression of not only NLRP3 but also pro- IL-1β (Sutterwala, Haasken et al. 2014). One of two main functions of Signal 1 is to upregulate the expression of NLRP3, caspase-1 and pro-IL-1β which will together form the inflammasome complex (Swanson, Deng et al. 2019). The other function of priming is to induce the post-translational modifications of NLRP3 in order to stabilize it. This stabilization enables NLRP3 to be in an inactive auto-suppressed state but also signal-competent (Swanson, Deng et al. 2019).

PAMP or DAMP signals provide activation signal, Signal 2, in order to trigger the activation of NLRP3 itself (Sutterwala, Haasken et al. 2014), initiate the assembly of inflammasome complex (Man and Kanneganti 2015), and subsequent activation of caspase-1 (Sutterwala, Haasken et al. 2014) as demonstrated in Figure 1. Upon formation of inflammasome, pro-caspase-1 protein oligomerize, their close proximity drives their auto-proteolytic cleavage and thereby their activation. Once each Caspase-1 is activated, these cysteine-dependent proteases cleave pro-inflammatory cytokines IL-1β and IL-18 into mature forms. Subsequent release of these active inflammatory cytokines induces more inflammatory response within their microenvironment. On the other hand, once activated, Caspase-1 may cause inflammatory cell death, named as pyroptosis (Guo, Callaway et al. 2015).

Several previous studies provided evidence supporting association of the aberrant NLRP3 inflammasome activation with several autoinflammatory, autoimmune, inflammatory and metabolic disease pathogenesis. Therefore, NLRP3 inflammasome activation should be highly regulated in order for preventing excessive inflammation along with damage to host (Jo, Kim et al. 2015).



* *Created with BioRender.*

Figure 1.: NLRP3 inflammasome activation: *NLRP3 inflammasome complex gets activated by sensing PAMPs and DAMPs. Inflammasome complex is comprised of NLRP3, ASC and Caspase-1 proteins and once it is activated, Caspase-1 of the complex cleaves precursor of IL-1β and IL-18 within the cell. Following this cleavage, mature IL-1β and IL-18 are secreted from the cell. Additionally, activated Caspase-1 protein might also leads pyroptotic cell death through Gasdermin D.*

2.9. Pyroptosis

Pyroptosis is a type of programmed inflammatory cell death (Jo, Kim et al. 2015). Pyroptosis is characterized cell swelling and later lysis of the cell resulting in releasing cellular contents into the environment (He, Hara et al. 2016). This inflammasome-dependent cell death causes release of DAMPs in order to further induce inflammation (Guo, Callaway et al. 2015). During pyroptosis, caspase-1 activation results in an increase in permeability of the membrane

and also calcium influx. This leads to lysosomal exocytosis followed by secretion of lysosomal proteins (Jo, Kim et al. 2015).

The activation of multiprotein complex inflammasome results in cleavage of cytokines promoting pyroptosis. Activated caspase-1 in inflammasome activation results in cleavage of Gasdermin-D, which in turn results in cell death (He, Hara et al. 2016). This process is regulated via N-terminal domain of GSDMD (Gasdermin D) through pore formation within the plasma membrane. Simultaneously, through potassium efflux, NLRP3 inflammasome is activated (Yang, Wang et al. 2019).

LPS (lipopolysaccharide) detection in cytosol causes activation of downstream caspases followed by cleavage of Gasdermin D and insertion of it within the membrane in order to form pores within the membrane which contributes to pyroptosis (Swanson, Deng et al. 2019). ATP is also an inducer of pyroptosis (Jo, Kim et al. 2015).

2.10. Diethyl Maleate

Diethyl maleate (DEM) is an electrophilic reagent that induces accumulation of Nrf2 (Kobayashi, Suzuki et al. 2016). It has $C_8H_{12}O_4$ molecular formula and 172.18 g/mol formula weight (Sigma-Aldrich). DEM is categorized as Nrf2 activator (Harada, Kanayama et al. 2011).

A number of studies showed anti-inflammatory effect of DEM *in vitro* and *in vivo*. In an *in vivo* study of Nathen *et.al.*, it has been shown that pretreatment of rats via DEM has been able to setback the LPS induction in lungs. It has been demonstrated that pretreatment of DEM decreases neutrophil accumulation and lung injury in animal models of the study, therefore, it has been determined to be reversing inflammation against endotoxin injury (Nathens, Marshall et al. 1996).

NO synthase, a mediator that is known to produce nitric oxide, is a significant regulator of inflammation. In a study of Kang *et.al.*, they have reported that DEM is a promising repressive agent as it might be an important inhibitor of iNOS expression in LPS-induced macrophages in their animal models. It has been demonstrated that toxicity of LPS has been inhibited through inhibition of NO synthase presumably due to pretreatment by DEM in endotoxic animal models (Kang, Pak et al. 1999).

Last but not least, treatment of cells by DEM has been shown to result in reduction of NF-kB activation in astrocytes in a study investigating brain inflammation (Kano, Choi et al. 2019), leading to the inference that DEM is a promising agent as it reduces activation of NF-kB, as it is known to induce expression of pro-inflammatory genes. However, DEM has not been discovered to repress NLRP3 inflammasome activation, yet.

Effects of Nrf2 inducing compounds has been stated to inhibit inflammasomes and inflammation as potential compounds to use for their anti-inflammatory characteristics (Hennig, Garstkiewicz et al. 2018). A number of stress response pathways within cells are activated upon exposure to varying chemicals. The reason under the regarding activation is to induce repair mechanisms of cells. On the other hand, it may lead cells to adapt the circumstances (Bischoff, Kuijper et al. 2019). Nrf2 stands for Nuclear factor-erythroid-2-related factor 2. In oxidative stress related states within the cell, the main regulator is Nrf2; and both activation and Nrf2 level are regulated via Keap1 (Silva-Islas and Maldonado 2018). Activation of Nrf2 is a defense mechanism as a response to the toxicity of oxidative stress and inflammation to cell types including neural cells, glia and endothelial cells (Genc and Genc 2009). In a basic understanding, in a situation of oxidative stress within the cell, following the Nrf2 activation, Nrf2 target genes are induced by Nrf2 where products of these genes necessitate in cell protection (Silva-Islas and Maldonado 2018). It has been demonstrated that accumulated Nrf2 binds to the regulatory element of related inflammatory cytokines genes and consequently decreases production of these cytokines. Also, proinflammatory cytokine gene transcription is inhibited via Nrf2 (Kobayashi, Suzuki et al. 2016). It has also been shown that DEM induces a number of proteins with antioxidant properties upon a precise dependence on Nrf2. This characteristic has been demonstrated in mouse macrophages (Harada, Kanayama et al. 2011). Diethyl Maleate has been shown in RAW264.7 cells to increase Nrf2 levels by interfering reactive cysteine residues of Keap1 (Iso, Suzuki et al. 2016). More specifically, DEM has been demonstrated to act on Cys151 of Keap1. Along with leading upregulation of Nrf2 accumulation in the nucleus, also basal cytoplasmic Nrf2 levels are demonstrated to increase. However, Nrf2 regulatory proteins including Keap1 is determined not to be affected by DEM introduction in terms of either their levels or interactions via DEM (Iso, Suzuki et al. 2016).

3. MATERIALS AND METHODOLOGY

3.1. Research Type

This study is an *in vitro* study.

3.2. Time and Place of the Study

This study was conducted at İzmir Biomedicine and Genome Institute, between December 2018 and December 2019.

3.3. Research Material

N9 mouse microglial cell line, provided by Dr. Paola Ricciardi-Castagnoli (Cellular Pharmacology Center, Italy), was used.

3.4. Variables of the Study

LPS/ATP and Diethyl Maleate (DEM) were the independent variables of the study. On the other hand, cell viability and death rates, ROS measurements, mRNA and protein levels of IL18/IL1β inflammatory cytokines and cell death type are the dependent variables in this study.

3.5. Tools for Data Collection

Microplate reader Varioskan Flash (Thermo Scientific, USA)

Microplate reader MultiSkan Go spectrophotometer (Thermo Scientific, USA)

Centro XS3 lb 960 microplate luminometer (Berthold Technologies, Germany)

Fluorescent microscope Olympus IX-71 (Olympus, Japan)

PrestoBlue™ Cell Viability Reagent (Invitrogen, USA) – REF A13262 – LOT 1922837

Quantikine ELISA Mouse IL-1β Immunoassay KIT (R&D Systems, USA) – Catalog no. MLB00C – Lot P221992

MN DNA, RNA and protein purification kit (MACHEREY-NAGEL, Germany) – REF 740955.50 – LOT 1810/005

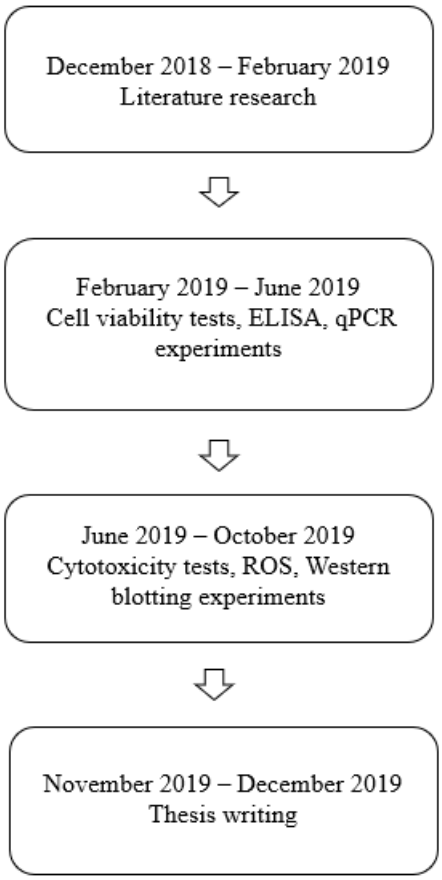
High Capacity cDNA Reverse Transcription Kit (AppliedBiosystems, Thermo, USA) – REF 4308228

Caspase-Glo-1 Inflammasome Assay (Promega, USA) – REF G9951 – LOT 0000350984

Cytotoxicity Detection – Lactate Dehydrogenase (LDH) Kit (Roche, Germany) – REF 11644793001 – LOT 35645500

MitoSOX^{TM} Red mitochondrial superoxide indicator (Molecular Probes, Invitrogen, USA) – REF M36008 – LOT 2037343

3.6. Study Plan and Calendar



3.7. Limitations of the study

As the conducted thesis is an *in vitro* study, the results should be supported and confirmed via *in vivo* experiments.

3.8. *In vitro* Experiments

N9 microglial cells were treated via ultra-pure LPS (1000ng/ml) (from E. coli 0111: B4) (InvivoGen, USA) for 4 hours, followed by 1-hour incubation with 5 mM ATP (adenosine 5′-triphosphate) (Sigma-Aldrich, USA). Prior to inflammasome activation via LPS and ATP, N9 cells were treated via 50, 100 and 250 μM doses of Diethyl Maleate for 1 hour.

3.8.1. Cell culture

Mouse N9 microglial cell line was provided by Dr. Paola Ricciardi-Castagnoli (Cellular Pharmacology Center, Italy). N9 microglia cell line originates from mouse brain and accordingly microglia are known to be similar to primary mouse microglia in many aspects (Stansley, Post et al. 2012).

Maintenance of N9 cell line was accomplished via RPMI 1640 (Sigma-Aldrich, USA) medium consisting of 10% Fetal Bovine Serum (FBS), a growth supplement, 2 mM L-Glutamine, 100 U/ml Penicillin and 100 μg/ml Streptomycin (P/S) under 37^{o}C and 5% CO_2 incubation conditions. The experiments were held with RPMI 1640 medium without P/S or FBS.

3.8.2. Determination of non-toxic doses of DEM by PrestoBlue viability assay

PrestoBlue^{TM} Cell Viability Reagent (Invitrogen, USA) was used to detect cytotoxicity of doses of DEM to be used. 1 x 104 cells/well were seeded to 96-well plate with 200 μl of final well volume and incubated overnight under 37^{o}C and 5% CO2 incubation conditions. After overnight incubation, microglial cells were pretreated with Diethyl Maleate with 0, 25, 50, 100, 250, 500 and 1000 μM concentrations for 1 hour. Next, the cells were treated with cell medium for a total of 5 hours to complete our 6-hour treatment model. After the treatment, cell culture media was collected, and PrestoBlue was added according to the given protocol. Absorbance

was measured according to manufacturer's instructions at 535 nm excitation and 615 nm emission wavelengths with microplate reader Varioskan Flash (Thermo Scientific, USA). Cell viability is expressed of reducing ability of viable cells to conduct a quantitative measurement of viability of cells, when PrestoBlue is introduced to cell environment, its nonfluorescent blue color changes into fluorescent red color due to reducing activity of proliferative cells. Measurement was performed as a percentage calculated by the following formula:

$$\text{Cell viability} = (OD_{Sample})/ (- OD_{Control}) * 100.$$

3.8.3. Determination of IL-1β cytokine level by ELISA Assay

N9 microglial cells were seeded into 96-well plate with 5 x 10^4 cells/well density with 200µl of final well volume and incubated overnight under 37ºC and 5% CO_2 incubation conditions. After overnight incubation, microglial cells were pretreated with Diethyl Maleate with 50, 100 and 250 µM concentrations for 1 hour. Next, the cells were treated with LPS for 4 hours and ATP for 1 hour. After the treatment, cell supernatant was collected. Levels of IL-1β, pro-inflammatory cytokines that are secreted after inflammatory stimuli were measured by using Quantikine ELISA Mouse IL-1β Immunoassay KIT (R&D Systems, USA) according to the manufacturer's protocol. The absorbance values were determined at 450 nm with 570 nm reference absorbance using microplate reader MultiSkan Go spectrophotometer (Thermo Scientific, USA). The related protein levels were detected depending on the standard graph plotted from protein standards of IL-1β.

3.8.4. Determination of IL-1β, IL-18 and NLRP3 at gene level by quantitative RT-PCR

N9 microglial cells were seeded into T25 cell culture flasks with 1 x 10^6 cells density and incubated overnight under 37ºC and 5% CO_2 incubation conditions. After overnight incubation, microglial cells were pretreated with Diethyl Maleate with 50, 100 and 250 µM concentrations for 1 hour. Next, the cells were treated with LPS for 4 hours and ATP for 1 hour. After the treatment, cells were scrapped from flasks via cell scrapers and cells were collected. For mRNA quantification in *in vitro* experiments, total RNA was isolated from cells using MN DNA, RNA and protein purification kit (MACHEREY-NAGEL, Germany) according to manufacturer's instructions. RNA purity and concentrations within the isolated sample were determined via

Nanodrop. cDNAs were synthesized by High Capacity cDNA Reverse Transcription Kit (AppliedBiosystems, Thermo, USA) using 2 μg of total RNA with random hexamer primer and was amplified using the mouse primers for target genes of proinflammatory cytokines, IL-1β, IL-18 and NLRP3. SYBR green was used within GoTaq qPCR Master Mix (Promega, USA). For normalization of mRNA data, endogenous Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. $2^{-\Delta Ct}$ formula was used to determine relative expression levels.

Tablo 1: List of primers

IL-1β	F	5'- GTGCTCATGTCCTCATCCTG-3'
	R	5'- CACAGCAGCACATCAACAAG-3'
IL-18	F	5'-CTTTGGAAGCCTGCTATAATCC-3'
	R	5'- GGTCAAGAGGAAGTGATTTGGA-3'
NLRP3	F	5'-TGCCTGTTCTTCCAGACTGGTGA-3'
	R	5’-CACAGCACCCTCATGCCCGG-3'
GAPDH	F	5’-ACCACAGTCCATGCCATCAC-3’
	R	5’-TCCACCACCCTGTTGCTGTA-3’

3.8.5. Determination of NLRP3 at protein level by Western Blotting

In order to demonstrate the inflammasome activation in microglial cells induced with LPS+ATP, 3 x 10^6 cells were seeded onto petri dishes and incubated overnight under 37^{o}C and 5% CO_2 incubation conditions. After overnight incubation, treatment of cells was accomplished via DEM, LPS and ATP. Later on, the cells were harvested via scrapping. Total protein was isolated from cells and lysed via RIPA lysis buffer comprised of 50mM Tris-HCl, pH 7.4, 150mM NaCl, 0.25% deoxycholic acid, 1% Nonidet P-40, 1mM EDTA including the protease and phosphatase inhibitor (Thermo Scientific, USA) extracted proteins were stored at −80◦C.

Equal amounts of proteins were loaded and separated with 8, 10 or 15% SDS-PAGE and were transferred onto polyvinylidene fluoride (PVDF) membranes (Sigma-Aldrich, USA). The membranes were blocked via BSA or milk with required percentages within TBST washing

solution, and membranes were incubated overnight at 4°C with primary antibodies of interested cytokines according to manufacturer's protocol. NLRP3 antibody used was anti-mouse (Adipogen) and Actin antibody (Abcam) was anti-rabbit. Membranes were then incubated with the horseradish peroxidase (HRP)-conjugated secondary antibodies (mouse or rabbit) accordingly. The antigen–antibody complex was detected by chemiluminescence using the Supersignal West Dura ECL reagent (Thermo Scientific, USA) and images was captured. Afterwards, the band densities were analyzed with ImageJ 1.51n and normalized to β-actin as loading control. Protein levels of inflammatory proteins NLRP3, Caspase-1 and IL-1β was then detected to be enhanced with LPS/ATP treatment indicating inflammasome induction; the proteins were then detected to be decreased with DEM suggesting a supressive effect of DEM.

Tablo 1: List of antibodies

NLRP3	Adipogen	Ag-20b-0014-c100	Anti-Mouse
β-actin	Abcam	Ab-8727	Anti-Rabbit

3.8.6. Determination of Caspase-1 by Caspase-1 activity assay

An important factor for inflammasome activation is the Caspase-1 in microglial cell population. To determine Caspase-1 activity, 1 x 10^4 cells/well were seeded to 96-well plate with 200 µl of final well volume and incubated overnight under 37ºC and 5% CO_2 incubation conditions. After overnight incubation, DEM pretreatment and LPS+ATP induction were achieved. Following the treatment, supernatant of the samples was taken to determine Caspase-1 activity via luminometric Caspase-Glo-1 Inflammasome Assay (Promega, USA) according to manufacturer's protocol. The treated samples were measured with Centro XS3 lb 960 microplate luminometer (Berthold Technologies, Germany).

3.8.7. Determination of reactive oxygen species by MitoSOX staining

1 x 10^4 cells/well were seeded to 96-well plate with 200 µl of final well volume and incubated overnight under 37ºC and 5% CO_2 incubation conditions. After overnight incubation, cells were treated via DEM, LPS and ATP to determine ROS produced by the mitochondria of the cells to detect the health status of the cells upon inflammasome induction. MitoSOX™ Red mitochondrial superoxide indicator (Molecular Probes, Invitrogen, USA) was diluted and added

onto cells with 5 μM and incubated for 15 minutes according to manufacturer's instructions. The absorbances of the cultured samples were determined via microplate reader Varioskan Flash (Thermo Scientific, USA). Additionally, fluorescence images of the treated cells were taken via inverted fluorescent microscope Olympus IX-71 (Olympus, Japan).

3.8.8. Determination of cell death by PI Staining

N9 microglial cells were seeded into 96-well plate with 1 x 10^4 cells/well density with 200μl of final well volume and incubated overnight under 37°C and 5% CO_2 incubation conditions. After overnight incubation, cells were treated via DEM, LPS and ATP. After the treatment, Propidium iodide staining was used to detect cell death. PI (Sigma-Aldrich, USA) (50 μg/ml) was added into the culture media and was incubated for 15 minutes. Stained cells were observed using inverted fluorescent microscope Olympus IX-71 (Olympus, Japan). PI positive cells was then counted using ImageJ 1.51n software and data was presented as percentage of PI positive cells.

3.8.9. Determination of pyroptotic cell death by LDH Assay

Cytotoxicity Detection – Lactate Dehydrogenase (LDH) Kit (Roche, Germany) was used to quantify cell death. 1 x 10^4 cells/well were seeded to 96-well plate with 200 μl of final well volume and incubated overnight under 37°C and 5% CO_2 incubation conditions. After overnight incubation, microglial cells were pretreated with Diethyl Maleate with 50, 100 and 250 μM concentrations for 1 hour. Next, the cells were treated with LPS for 4 hours and ATP for 1 hour. After the treatment, cell culture media was collected, and LDH activity was quantified according to the given protocol. Absorbance was measured at 492 nm (reference wavelength: 630 nm) on microplate reader MultiSkan Go spectrophotometer (Thermo Scientific, USA). Cytotoxicity is expressed as a percentage of the total amount of LDH released from lysed cells, calculated by the following formula:

$$\text{Cytotoxicity} = (OD_{Sample} - OD_{Control}) / (OD_{MaximalRelease} - OD_{Control}) * 100.$$

4. RESULTS

4.1. Determination of toxic doses of Diethyl Maleate by PrestoBlue viability assay

Varying doses of Diethyl Maleate ranging from 25 to 1000 μM with an addition of non-treated group were given to microglial cells that had been seeded 1 x 10^4 cells/well in 96-well plate and in order to determine NLRP3 inflammasome activation repressing but non-toxic DEM doses. Viable cells were detected via PrestoBlue™ Cell Viability Reagent (Invitrogen, USA). As seen in Figure 2, following the treatment of cell via 50 μM DEM, cell viability was observed to be 104 ± 0,8312 standard deviation and P<0,0303. Likewise, 100 μM DEM treated cells were with 108,3 ± 1,261 standard deviation and P<0,0043 depending on Mann-Whitney test. It was concluded that upon pretreatment via 50, 100 and 250 μM DEM cell viability of cells was appropriate to continue with.

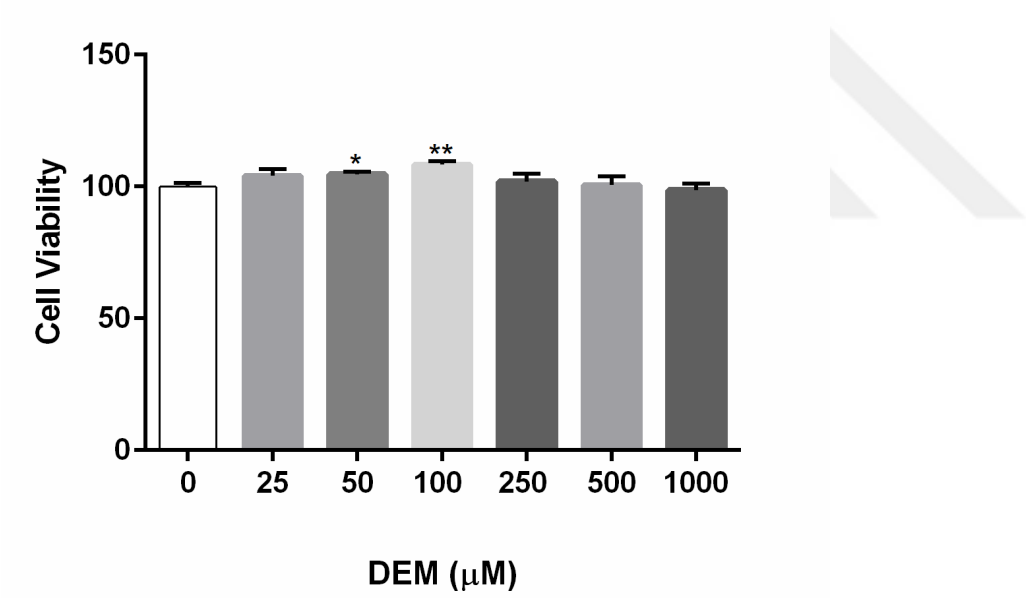


Figure 2.: Effect of variable doses of DEM on cell viability. *Viability of varying concentrations of DEM was investigated in order to determine the optimal nontoxic doses. Results were given as mean ±SD.*

4.2. Effect of Diethyl Maleate on protein level of IL-1β cytokine by ELISA Assay

Following the treatment of N9 microglial cells with DEM and LPS+ATP, effect of regarding treatment on levels of IL-1β cytokine was analyzed via ELISA assay. It was

demonstrated that after treatment via LPS+ATP, IL-1β cytokine level significantly increased with mean as mean with standard deviation 408,1 ± 32,52 pg/ml and P<0,0238, as seen in Figure 3. Following the treatment of N9 microglial cells via 50 μM DEM, IL-1β cytokine was observed to statistically significantly decrease with 50,43 ± 1,708 pg/ml standard deviation and P<0,0022. Likewise, 100 μM DEM treated cells were observed to significantly decrease IL-1β cytokine levels with 9,602 ± 0,7752 pg/ml, P<0,0022 and 250 μM DEM with 0,4955 ± 0,1472 pg/ml standard deviation and P<0,0238 depending on Mann-Whitney test. It was concluded that upon pretreatment N9 microglia via DEM, IL-1β cytokine level has been significantly decreased in a dose dependent manner in cells induced with LPS and ATP treatment.

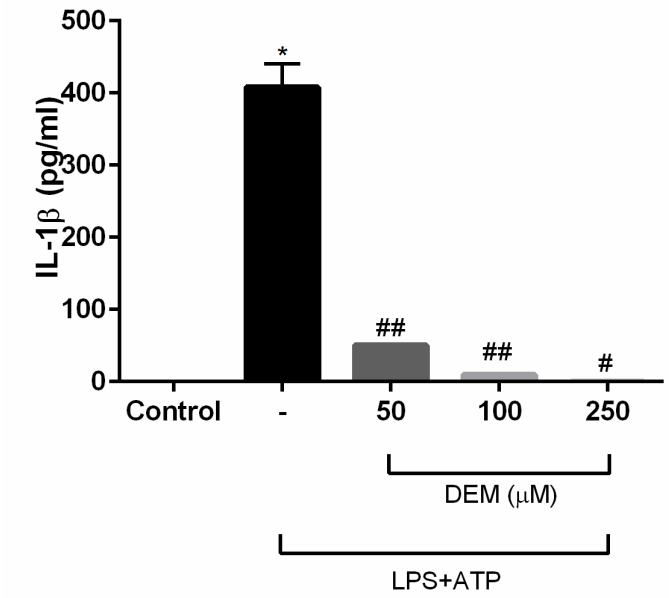


Figure 3.: Dose dependent effect of DEM on IL-1β cytokine levels*. Cytokine levels of IL-1β were determined to decrease significantly upon DEM pretreatment, after inflammasome induction by LPS+ATP. Results were given as mean ±SD.*

4.3. Effect of Diethyl Maleate on mRNA expression levels of IL-1β, IL-18 and NLRP3 genes by qPCR

Following the treatment via DEM and LPS+ATP, the changes in mRNA expression levels of the proteins secreted in inflammasome pathway, IL-1β, IL-18 and NLRP3, were investigated. As seen in Figure 4, it was demonstrated that after LPS+ATP treatment, ***(A)*** IL-1β mRNA level

of the cells were significantly increased as mean with standard deviation 301,6 ± 52,59 and P<0,0095. Following the treatment of cell via 50 μM DEM, mRNA level was observed to decrease with 49,89±8,599 standard deviation. Likewise, 100 μM DEM treated cells were observed to decrease expression levels of IL1β with 21,63±1,926 and 250 μM DEM with 6,083±1,283 standard deviation. The mRNA levels have been determined to be significantly different from regarding comparison with P<0,0095 depending on Mann-Whitney test.

It was demonstrated that after LPS+ATP treatment, ***(B)*** IL-18 mRNA levels of the cells were significantly increased as mean with standard deviation 1,711 ± 0,1760 and P<0,0002. Following the treatment of cell via 50 μM DEM, mRNA level was observed to decrease with 0,7682±0,05218 standard deviation and P<0,0001. Likewise, 100 μM DEM treated cells were observed to decrease expression levels of IL18 with 0,6022±0,02903 and P<0,0001; and 250 μM DEM with 0,5643±0,5855 standard deviation and P<0,0001. The mRNA levels have been determined to be significantly different from regarding comparison with P<0,0001 depending on Mann-Whitney test.

It was demonstrated that after LPS+ATP treatment, ***(C)*** NLRP3 mRNA level of the cells were significantly increased as mean with standard deviation 5,057±0,8672 and P<0,0001. Following the treatment of cell via 50 μM DEM, mRNA level was observed to decrease with 2,494±0,1663 standard deviation and P<0,0149. Likewise, 100 μM DEM treated cells were observed to decrease expression levels of IL18 with 2,113±0,2631 and P<0,0017; and 250 μM DEM with 2,077±0,1749 standard deviation and P<0,0001. The mRNA levels have been determined to be significantly different from regarding comparison with P<0,0011 depending on Mann-Whitney test.

As seen in Figure 4, DEM was concluded to significantly decrease the mRNA levels of IL-1β, IL-18 and NLRP3 genes that had increased via inflammasome activation in microglial cells induced via LPS+ATP.

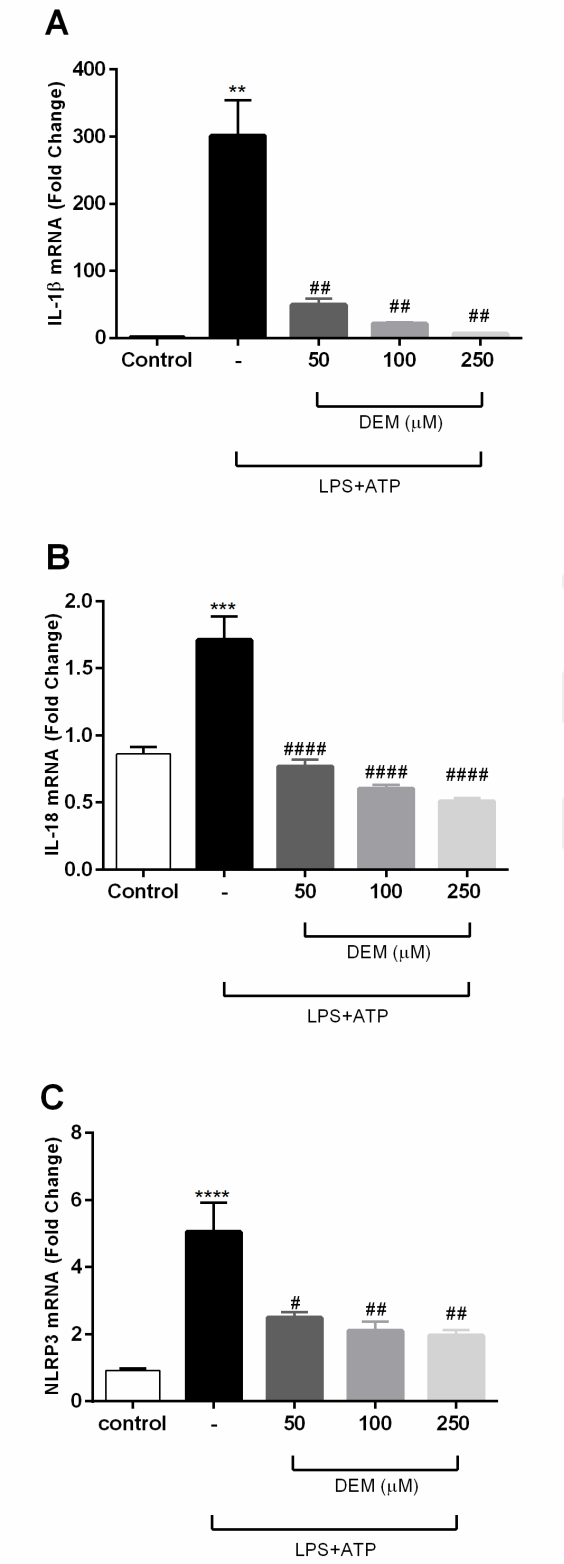


Figure 4.: Dose dependent effects of DEM on expression levels of mRNA of indicator genes of inflammasome, (A) IL-1β, (B) IL-18 and (C) NLRP3*. Expression levels of IL-1β, IL-18 and NLRP3 genes were determined to decrease significantly upon DEM pretreatment, after inflammasome induction by LPS+ATP. Results were given as mean ±SD.*

4.4. Effect of DEM on protein level NLRP3 by Western blotting

Effect of DEM on NLRP3 protein level as an important indicator of inflammasome activation in microglial cells was determined via immunoblotting. As seen in Figure 5A, increase with LPS+ATP treatment and decrease upon DEM pretreatment were visualized as bands of proteins. As seen in Figure 5B, after LPS+ATP treatment, NLRP3 level as band intensity was significantly increased as mean with standard deviation 1,903 ± 0,2577 and P<0,0357. Following the pretreatment of microglial cells via DEM, NLRP3 protein level was observed to decrease with 1,084 ± 0,1490 standard deviation and P<0,0357depending on Mann-Whitney test. It was concluded that upon pretreatment via DEM, NLRP3 protein band intensity has been significantly decreased in microglial cells induced with LPS and ATP treatment.

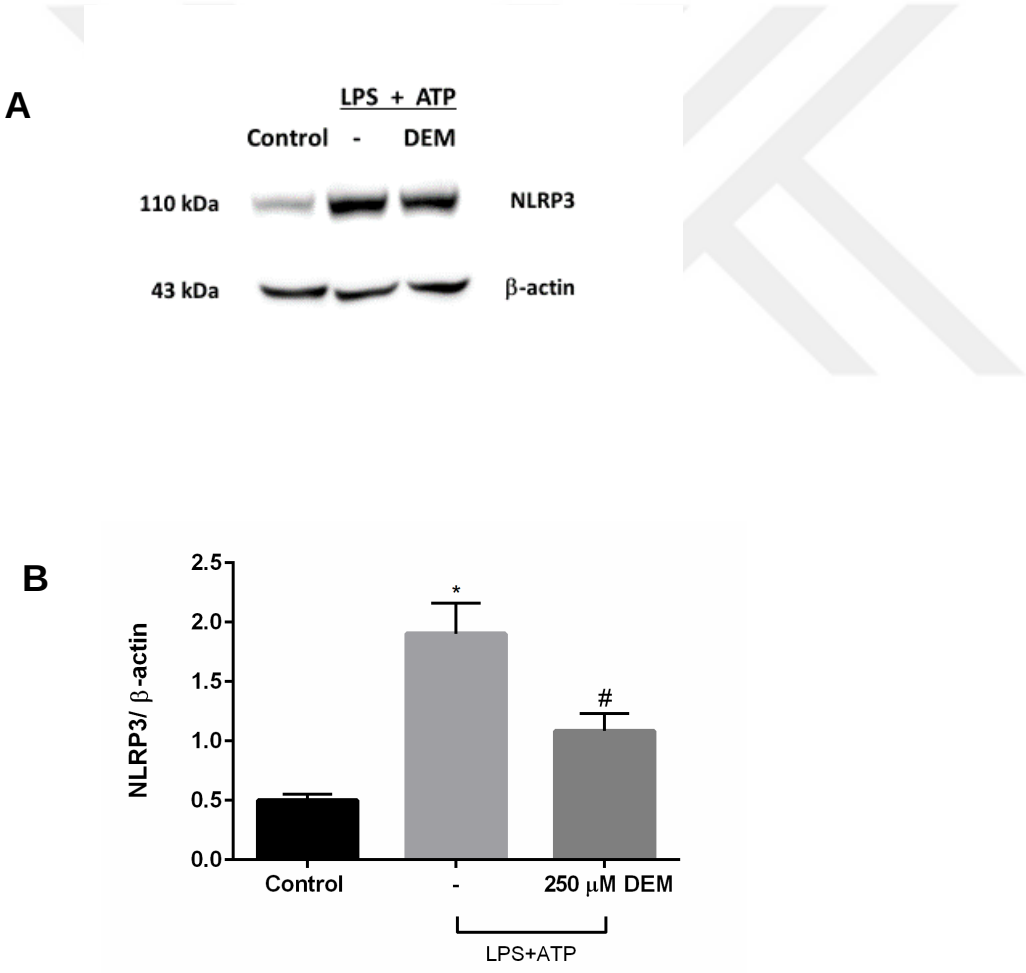


Figure 5.: Effect of DEM on NLRP3 protein level. *NLRP3 protein level was determined to decrease significantly upon DEM pretreatment, after inflammasome induction by LPS+ATP. Results were given as mean ±SD.*

4.5. Effect of DEM on Caspase-1 activity by activity assay

Activity of Caspase-1 which is an important indicator of inflammasome activation in microglial cells was determined via Caspase-1 activity assay. As seen in Figure 6, after LPS+ATP treatment, activity of Caspase-1 significantly increased as mean with standard deviation 225,7 ± 23,49. Following the pretreatment of microglial cells via DEM, Caspase-1 activity was observed to decrease with 118,3 ± 3,957 standard deviation and P<0,0079 depending on Mann-Whitney test. It was concluded that upon pretreatment via DEM, Caspase-1 activity has been significantly decreased in microglial cells induced with LPS and ATP treatment.

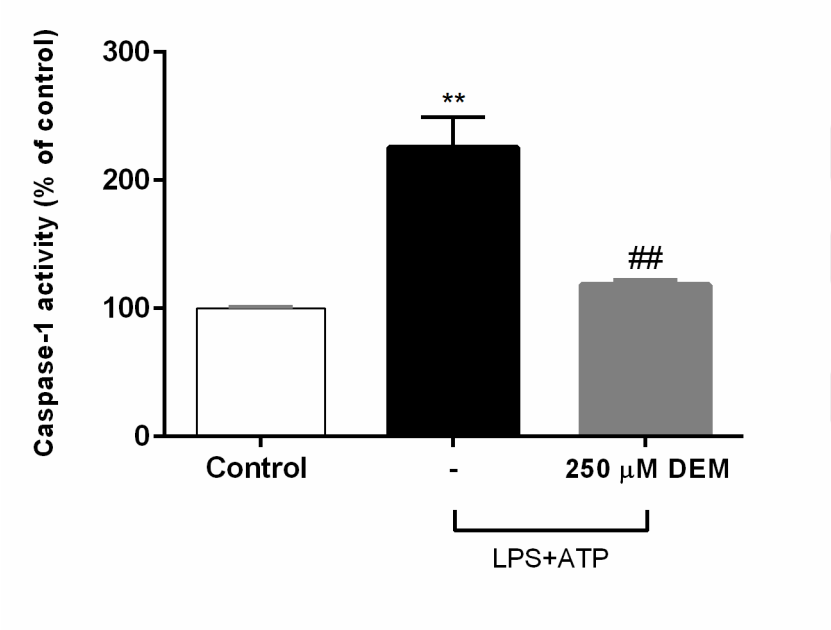


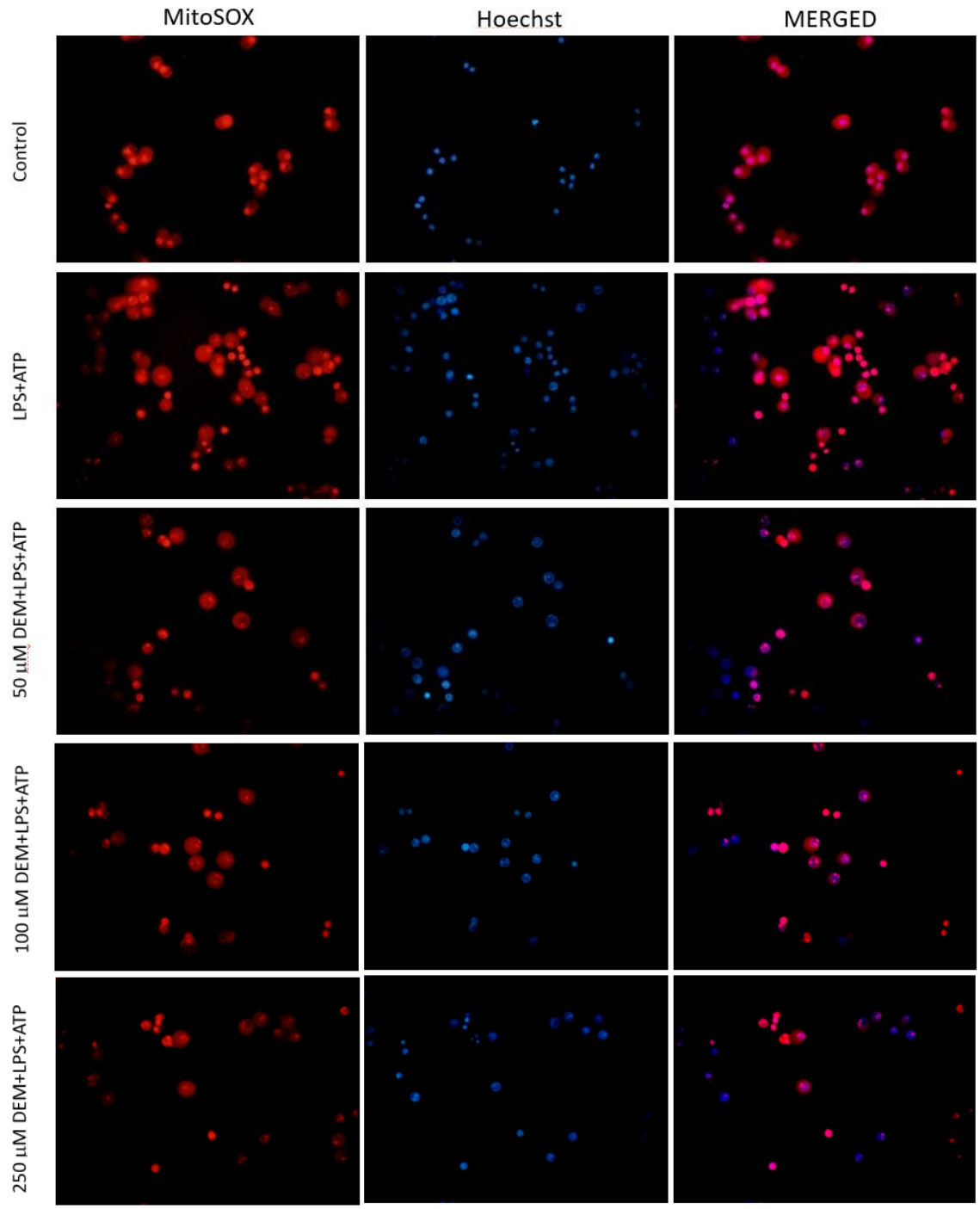
Figure 6.: Effect of DEM on Caspase-1 activity. *Caspase-1 activity was determined to decrease significantly upon DEM pretreatment, after inflammasome induction by LPS+ATP. Results were given as mean ±SD.*

4.6. Effects of DEM on mitochondrial ROS production by MitoSOX

The mitochondrial superoxide level which increases within the cell upon induction via LPS+ATP was investigated via MitoSOX. As seen in Figure 7, after LPS+ATP treatment, mitochondrial superoxide level was significantly increased as mean with standard deviation 11,53 ± 1,418 and P<0,0001. Following the treatment of cell via 50 μM DEM, superoxide level was observed to decrease with 6,646±0,5802 standard deviation and P<0,0030. Likewise, 100 μM DEM treated cells were observed to decrease levels with 4,302±1,325, P<0,0050 and 250

μM DEM with 3,134±1,169 standard deviation and P<0,0003 depending on Mann-Whitney test. It was concluded that upon pretreatment via DEM, mitochondrial ROS level has been significantly decreased in dose dependent manner in microglial cells induced with LPS and ATP treatment.

A


MitoSOX
Hoechst
MERGED
Control
LPS+ATP
50 µM DEM+LPS+ATP
100 µM DEM+LPS+ATP
250 µM DEM+LPS+ATP

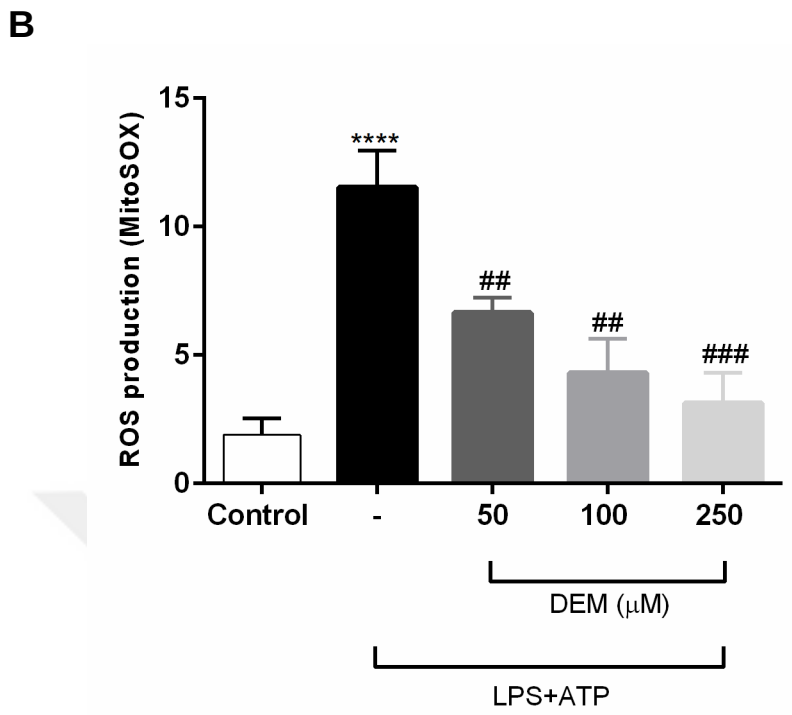
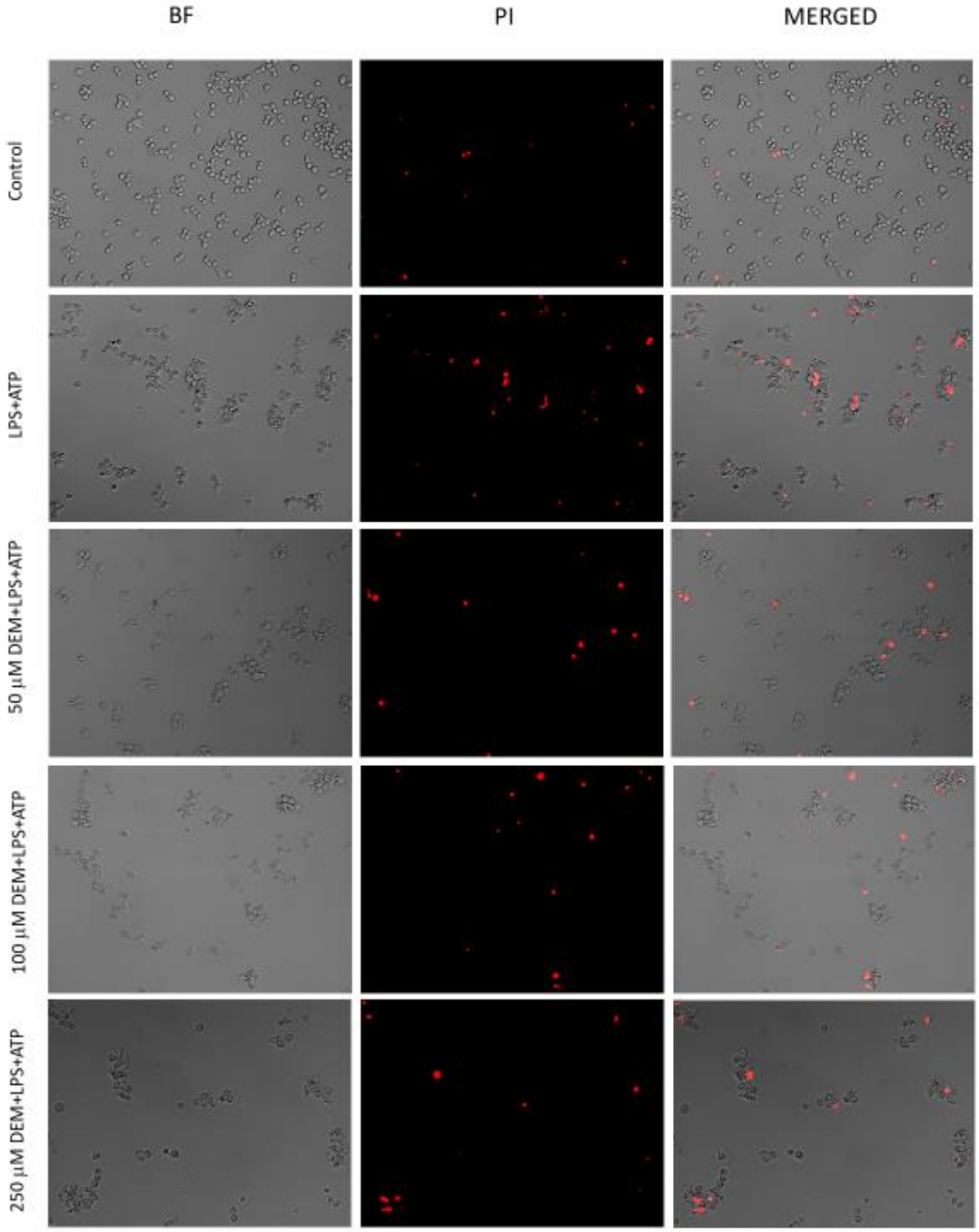


Figure 7.: Dose dependent effect of DEM on production of mitochondrial ROS. *Mitochondrial ROS levels were determined to decrease significantly upon DEM pretreatment, after inflammasome induction by LPS+ATP. Results were given as mean ±SD.*

4.7. Effect of DEM on pyroptotic cell death by PI staining

Microglia cells were stained via PI dye after LPS+ATP treatment and DEM pretreatment. As seen in Figure 8, after LPS+ATP treatment, Cell death was significantly increased as mean with standard deviation 20,79 ± 3,944 and P<0,0357. Following the treatment of cell via 50 μM DEM, cell death ratio was observed to decrease with 11,50±1,485 standard deviation and P<0,0357. Likewise, 100 μM DEM treated cells were observed to decrease levels with 10,60 ± 0,7327, P<0,0357 and 250 μM DEM with 6,068 ± 0,8984 standard deviation and P<0,0357 depending on Mann-Whitney test. It was concluded that upon pretreatment via DEM, cell death ratio has been significantly decreased in a dose dependent manner in microglial cells induced with LPS and ATP treatment

A


BF
PI
MERGED
Control
LPS+ATP
50 µM DEM+LPS+ATP
100 µM DEM+LPS+ATP
250 µM DEM+LPS+ATP

B

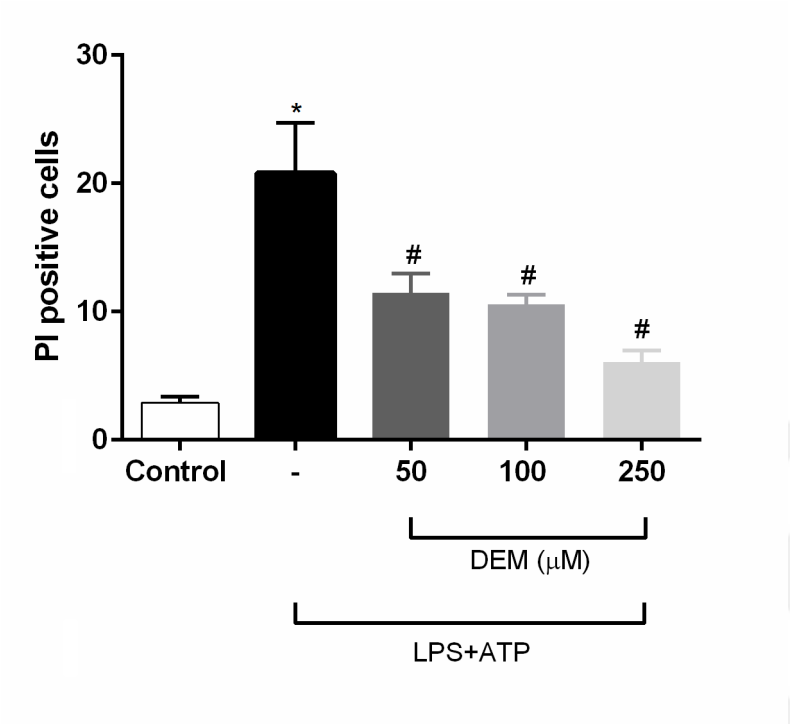


Figure 8.: Dose dependent effect of DEM on pyroptotic cell death via PI staining. *Pyroptotic cell death was determined to decrease significantly upon DEM pretreatment, after inflammasome induction by LPS+ATP. Results were given as mean ±SD.*

4.8. Effect of DEM on pyroptotic cell death by LDH cytotoxicity test

Microglia cell death after LPS+ATP treatment and DEM pretreatment were determined via LDH test. As seen in Figure 9, after LPS+ATP treatment, cell death significantly increased as mean with standard deviation 15,13 ± 0,9420. Following the treatment of cell via 50 μM DEM, cell death ratio was observed to decrease with 7,983 ± 0,6028 standard deviation. Likewise, 100 μM DEM treated cells were observed to decrease cell death with 3,814 ± 0,1418 and 250 μM DEM with 3,188 ± 0,1685 standard deviation with $P<0,0079$ depending on Mann-Whitney test. It was concluded that upon pretreatment via DEM, cell death has been significantly decreased in a dose dependent manner in microglial cells induced with LPS and ATP treatment.

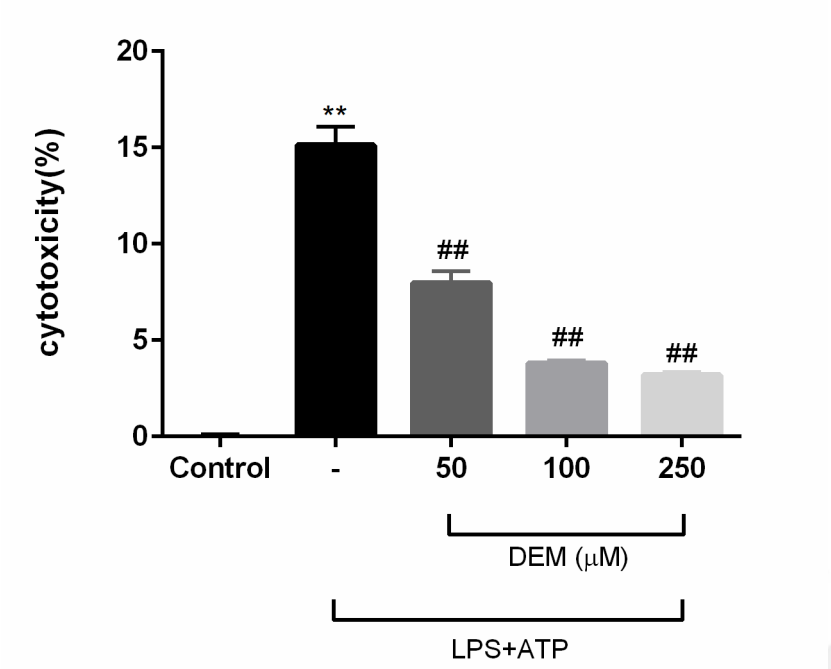


Figure 9.: Dose dependent effect of DEM on pyroptotic cell death via cytotoxicity test. *Pyroptotic cell death was determined to decrease significantly upon DEM pretreatment, after inflammasome induction by LPS+ATP. Results were given as mean ±SD.*

5. DISCUSSION

Inflammasome protein complexes are assembled in order to regulate host innate immune responses against damage to cells or infection (Yang, Wang et al. 2019). The conception of the importance of microglia in homeostasis has been well-established (Wlodarczyk, Holtman et al. 2017). A significant characteristic regarding microglia response is microglia are able to provide response not only to exterior signals but also to interior signals including stress signals and damaged cell signals (Aloisi 2001). As microglia have role in inflammasome activation, unnecessary activation of inflammasome and/or redundant activation are known to contribute to neuroinflammatory and neurodegenerative diseases (Wlodarczyk, Holtman et al. 2017). Regarding the importance of functional microglia and contribution of excess inflammation to several neurodegenerative disease including Alzheimer's disease, Parkinson's disease, Schizophrenia and Multiple Sclerosis (Hanisch and Kettenmann 2007), inhibition of NLRP3 inflammasome activation would be a promising idea in stabilization of homeostasis in the CNS and thus in repression the progression and inflammatory effects of neurodegenerative diseases.

NLRP3 inflammasome is the well-defined sensor molecule of inflammasome and is described as a key sensor molecule of cellular oxidative stress and alterations in homeostatic conditions (Pellegrini, Fornai et al. 2019). Once inflammasome multi-protein complex is formed, it recruits Pro-caspase-1 through its adaptor protein (ASC) in order to cleave precursors of IL-1β and IL-18 pro-inflammatory cytokines into their mature forms on the purpose of inducing inflammatory response within cells which also results in caspase-dependent cell death form, pyroptosis (Yang, Wang et al. 2019).

In our thesis Project, NLRP3 inflammasome activation was induced via 1 μg/ml LPS followed by 5mM ATP. Regarding model of NLRP3 inflammasome stimulation was based on previous studies of stimulation by 1 μg/ml LPS and 5 mM ATP of BV-2 cells due to the fact that BV-2 cells are also murine microglial cell line (Cai, Kong et al. 2016). In a study of NLRP3 inflammasome in N9 murine microglial cell line, LPS+ATP model of inflammasome induction has been conducted as with 1μg/ml LPS for 4 hours and 5mM ATP for 1 hour (Arioz, Tastan et al. 2019). LPS provides the first signal (priming) required for inflammasome activation (Wolf, Boddeke et al. 2017), whereas ATP serves for NLRP3 inflammasome complex formation (Mao, Chen et al. 2013). Accordingly, we have performed NLRP3 inflammasome activation by treating N9 murine microglial cell line by 1 μg/ml LPS for 4 hours followed by 5mM ATP for 1 hour.

An important result of microglial response is secretion of proinflammatory cytokines including IL-1β and IL-18 through induction (Wolf, Boddeke et al. 2017). Presence of pathogen and danger signals activate NLRP3 inflammasome complex (Guo, Callaway et al. 2015). Following the activation of NLRP3 complex activation, mature Caspase-1 cleaves pro-IL-1β and pro-IL-18 into their mature forms which are then released from cells (Nizami, Hall-Roberts et al. 2019). As previously performed in BV-2 murine microglia (Cai, Kong et al. 2016) and in N9 murine microglia (Arioz, Tastan et al. 2019), in our studies, increase in pro-inflammatory cytokines IL-1β and IL-18 followed by NLRP3 inflammasome activation via LPS+ATP model has been confirmed in N9 microglia. A statistically significant increase in IL-1β and IL-18 cytokines has been demonstrated at mRNA level with qPCR. Additionally, regarding pro-inflammatory cytokines have been shown to increase via LPS+ATP induction at protein level by ELISA and Western immunoblotting techniques.

Pyroptosis, inflammatory type of cell death, is also induced via NLRP3 inflammasome activation. It is also known as Caspase-1-dependent cell death as pyroptosis is induced via activation of Caspase-1 followed by cleavage of Gasdermin-D (Nizami, Hall-Roberts et al. 2019). In regarding type of cell death, danger signals are sensed through NLRP3 sensor molecule and through the complex activation, and the maturation of pro-inflammatory cytokines, at the same time cell death is initiated through autocatalysis (Jo, Kim et al. 2016). Pyroptotic cell death upon LPS+ATP induction has been shown to increase in N9 microglia (Arioz, Tastan et al. 2019). Similarly, in our studies in N9 murine microglial cell line, it has been demonstrated by cytotoxicity test and PI staining techniques that following the activation of inflammasome, pyroptotic cell death is significantly increased via our LPS+ATP model. Accordingly, statistically significant increase in Caspase-1 activity in N9 cells upon inflammasome activation via LPS+ATP model has been shown by Caspase-1 activity assay.

Innate immunity is responsible for immune responses through recognition of DAMPs from host cells or PAMPs from pathogens. Following the recognition of exogenous or endogenous danger signals, innate immunity acts through NLRP3 inflammasome complex activation (Mamik and Power 2017). Mitochondrial ROS is produced upon activation and production is also capable of triggering NLRP3 inflammasome activation (Zhou, Yazdi et al. 2011). ROS are able to interfere the upstream NLRP3 pathway (Zhou, Yazdi et al. 2011). Mitochondrial membrane potential is also involved in this pathway, as in when the membrane potential is

altered, ROS production is triggered (Zhou, Yazdi et al. 2011). In a study of inflammasome investigating ROS and NLRP3 inflammasome relation, ROS production was induced by interfering upstream of the respiratory pathway in order for altered mitochondrial membrane potential (Zhou, Yazdi et al. 2011). They have shown that along with increase in ROS, IL-1β presence in THP1 macrophage cell line supernatant increased. Therefore, NLRP3 inflammasome activation was increased (Zhou, Yazdi et al. 2011). In another study, a relation between mitochondrial ROS generation and NLRP3 inflammasome has been revealed in primary microglia cell line (Sarkar, Malovic et al. 2017). They have demonstrated that mitochondrial dysfunction has role in increase of IL-1β level and activation of NLRP3 inflammasome (Sarkar, Malovic et al. 2017). In our study in with LPS+ATP model induced NLRP3 inflammasome in N9 microglial cell line, we have demonstrated via MitoSOX staining that mitochondrial ROS generation has been statistically significantly increased upon induction; following the pretreatment via DEM, ROS levels have been determined to dose-dependently and statistically significantly decreased.

Diethyl Maleate is a known activator of Nrf2 and is an endogenous electrophilic reagent normally produced by macrophages (Kobayashi, Suzuki et al. 2016). There are limited studies on effect of DEM in peripheric inflammation. In an *in vivo* study of Nathens group, DEM was demonstrated to capable of reducing inflammatory response in LPS-induced acute injury of lung in rats with 1-hour DEM pretreatment. Therefore, they have concluded that DEM is a promising agent to reduce acute lung injury and other neutrophil mediated injuries in endotoxin (LPS)-stimulated inflammations (Nathens, Marshall et al. 1996). Likewise, DEM has been demonstrated to be protective against liver inflammation against both endotoxin and TNF stimulation. In the study, DEM has been determined to prevent LPS-stimulated both hepatocyte necrosis and apoptosis in rodent endotoxemia model against inflammatory stimuli (Jones, Fan et al. 1999). In a study of mouse primary glia and *in vitro* cells, the involvement of astrocytes and microglia in brain inflammation has been stated (Kano, Choi et al. 2019). Both are capable of sensing immune stimuli. In this study, it has been shown that DEM introduction leads to attenuation in activation of NF-κB in astrocytes, where a significant association of astrocytes and microglia activation is known (Kano, Choi et al. 2019).

DEM has been stated as a potent protective agent in an *in vivo* study of macrophages (Kang, Pak et al. 1999). It has been demonstrated to be an inhibitor of iNOS expression, where iNOS

(NO synthase) produces nitric oxide (NO). iNOS is known to be important in regulating inflammation. In LPS-treated mice, DEM has shown to inhibit upregulation of serum IL-1β levels. Also, they have demonstrated that toxic effects of LPS in endotoxemic mice models has been attenuated through inhibition of NO synthase due to DEM pretreatment (Kang, Pak et al. 1999).

DEM has been previously demonstrated to reduce inflammatory response against LPS-induction (Kobayashi, Suzuki et al. 2016) and against both endotoxin and TNF stimulation (Jones, Fan et al. 1999) and also microglia involvement has been stated (Kano, Choi et al. 2019). However, effects of DEM on NLRP3 inflammasome activation and regarding inflammasome response have not been investigated, yet. In our study, the novelty of the study is investigation of the repressing effects of Diethyl Maleate on LPS+ATP induced NLRP3 inflammasome in N9 microglia. N9 cells were induced via LPS+ATP to activate NLRP3 inflammasome. Levels of inflammasome activation markers NLRP3, Caspase-1, IL-1β, IL−18 have been investigated in mRNA and protein levels via qPCR, immunoblotting, ELISA and Caspase activity assay respectively. It has been determined that as their levels significantly increased upon LPS+ATP induction, and upon pretreatment via DEM, levels of regarding inflammasome markers have been decreased indicating protective effect of DEM in N9 microglial cell line. Upon pretreatment via DEM, IL-1β, IL-18 cytokine levels and NLRP3 statistically significantly decreased in a dose-dependent manner. We have also shown that Caspase-1 activity significantly increase via inflammasome activation by LPS+ATP treatment and upon DEM pretreatment, Caspase-1 activity significantly decreases. Therefore, in our study both levels of pro-inflammatory cytokines and inflammasome marker proteins are demonstrated to be decreased via pretreatment of DEM in N9 microglia cell line.

The protective and/or repressing effect of DEM in NLRP3 inflammasome activated pyroptotic cell death has not been discovered previously. In our study, we have demonstrated DEM's NLRP3 inflammasome repressing effect and decrease in pyroptotic cell death for the first time. We have demonstrated that pyroptosis has increased LPS+ATP induced N9 microglia and pyroptotic cell death level has statistically significantly and dose dependently decreased upon pretreatment via DEM.

Nrf2 pathway should be evaluated due to the fact that DEM acts through Nrf2 pathway. Nrf2 has been stated to repress inflammation through oxidative stress regulation (Kobayashi,

Suzuki et al. 2016). DEM is an important promising endogenous activator of Nrf2. As a response to oxidative stress, Nrf2 is capable of suppressing inflammatory responses through blockage of proinflammatory cytokines induced by LPS (Kobayashi, Suzuki et al. 2016). The path that Nrf2 transcription factor follows, to interfere cytokine upregulation is to bind to proximal regions of pro-inflammatory cytokine genes to inhibit expression (Kobayashi, Suzuki et al. 2016). Under normal conditions, Nrf2 level is stabilized at lower levels than its regulator proteins including Keap1. Once DEM is introduced, a significant increase in Nrf2 level is observed, resulting in an accumulation of Nrf2 within the nucleus (Iso, Suzuki et al. 2016).

The potential beneficial effects of Nrf2 inducing compounds has been stated as aforementioned compounds are more likely to inhibit inflammasomes and in accordance with this, they inhibit inflammation (Hennig, Garstkiewicz et al. 2018). A study of RAW264.7 cell line DEM, was demonstrated to cause a dramatic increase in Nrf2 protein (Iso, Suzuki et al. 2016). It has been also shown that DEM treatment upregulated and accumulated Nrf2 within the nucleus (Iso, Suzuki et al. 2016). LPS-induced upregulation of pro-inflammatory cytokines including IL-1β is attenuated via Nrf2 in a study of both *in vivo* murine and *in vitro* macrophages. DEM treatment to aforementioned models resulted in a significant inhibition of LPS-induced secretion of IL-1β and IL-6 (Kobayashi, Suzuki et al. 2016). In this thesis, Nrf2 pathway has not been investigated, however, it is to be investigated in the continuum of the project.

6. CONCLUSION AND FUTURE ASPECTS

In our project, effects of Diethyl Maleate on NLRP3 inflammasome activation in microglia has been investigated. In accordance with this purpose, LPS+ATP induced NLRP3 inflammasome model has been pretreated with DEM in N9 murine microglial cell line and its effects have been determined. Following the determination of non-toxic effective doses of DEM, repressive effects of DEM on inflammasome induced microglia have been demonstrated. NLRP3 inflammasome markers including proinflammatory cytokines IL-1β and IL−18, NLRP3 and Caspase-1 proteins have been demonstrated to be repressed upon DEM pretreatment of inflammasome induced N9 microglia. Consequently, 1-hour DEM pretreatment in LPS+ATP model inflammasome induction has been determined to repress IL-1β and IL−18 proinflammatory cytokines, mitochondrial ROS and Caspase-1 activity. Likewise, DEM has been demonstrated to significantly decrease inflammasome-related protein expression and also pyroptotic cell death. Therefore, Diethyl Maleate is a promising protective agent against NLRP3 inflammasome in microglial cells. Regarding the continuation of the study, Nrf2 pathway in integration of Diethyl Maleate's mechanism of action is to be investigated. Additionally, effects of Diethyl Maleate on NLRP3 inflammasome, in terms of inflammasome-related proteins, is to be investigated in further protein levels.

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