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Investigation of molecular mechanisms of macrophage activation

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ABBREVIATIONS

Aβ – amyloid beta
AD – Alzheimer's disease
ANOVA – Analysis of variance
APP – amyloid precursor protein
ASC – apoptosis associated speck-like protein containing a CARD
BSA – bovine serum albumin
CARD – caspase recruitment domain
Cyt D – cytochalasin D
DAMPs – damaged-associated molecular patterns
FBS – fetal bovine serum
GFP – green fluorescent protein
HaPyV – hamster polyomavirus
HFIP – 1,1,1,3,3,3-hexafluorisopropanol
HRP – horseradish peroxidase
IFN – interferon
IL – interleukin (for example, IL-1β, IL-6)
iPSC – induced pluripotent stem cells
KIPyV – Karolinska Institute polyomavirus
LDH – lactate dehydrogenase
LPS – lipopolysaccharide
MCPyV – Merkel cell polyomavirus
MeV – measles virus
MuV – mumps virus
N protein – nucleocapsid protein
NLPs – nucleocapsid-like particles
PAMPs – pathogen-associated molecular patterns
PBS – phosphate buffer saline
PI – propidium iodide
P/S – penicillin/streptomycin
PyV – polyomavirus
TNF-α – tumour necrosis factor alpha
TLR – toll-like receptor
Treg – regulatory T cells
VLPs – virus-like particles
WB – Western-blot

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INTRODUCTION

Macrophages are innate immune cells that respond to invaders immediately after infection (Mosser and Edwards, 2008). They reside in different body tissues and in addition to their defensive function these cells maintain the homeostasis (Varol *et al.*, 2015). Monocytes circulating in the blood differentiate to macrophages upon infection and then remove pathogens, cellular debris and alarm to other immune cells about danger together with tissue resident macrophages (Zhang *et al.*, 2021a). On infection site these cells cope with pathogens, secrete inflammatory molecules and chemokines to recruit different immune cells. Macrophages and dendritic cells travel to lymph nodes and present antigens to T lymphocytes (Muntjewerff *et al.*, 2020).

Inflammation is one of the major subjects under investigation related to macrophages. Chronic inflammation is linked to many illnesses (Parisi *et al.*, 2018). Macrophages as tissue resident cells contribute to the initiation and maintenance of the inflammation (Watanabe *et al.*, 2019). These cells attempt to remove pathogens and other harmful agents, like cholesterol crystals or misfolded protein aggregates, and restore homeostasis (Laskin *et al.*, 2011). However, long-lasting cell activation disturbs the balance of the immune response and macrophages can promote tissue damage.

Macrophage activation by various pathogen-associated molecular patterns (PAMPs), like lipopolysaccharide (LPS) and viral nucleic acids, is well studied (Mogensen, 2009). However, macrophage activation by endogenous molecules and environmental particles, for example, carbon, silica and asbestos nanoparticles, crystal microparticles (Mulay *et al.*, 2020), and protein aggregates (Aguzzi and O'Connor, 2010) is becoming increasingly studied due to their potential harmful effect on our health. Endogenous molecules, as well as PAMPs, can activate macrophages and cause inflammation. It was demonstrated that engineered nanoparticles used in the industry (electronic components, cosmetics, antimicrobial fabrics and sprays, sunscreens, cleaning products, etc.) are toxic to the cells and can mediate an inflammatory response (Savolainen *et al.*, 2010). They are transferred to our body via inhalation or through the skin. After recognition of these particles by macrophages, other immune cells are recruited to the site of inflammation. Continuous uptake of nanoparticles causes an uncontrolled inflammatory response leading to a tissue damage. Therefore, knowledge of mechanisms of cell activation would let us avoid uncontrolled inflammatory diseases.

The inflammasome is an intracellular pattern recognition receptor which can recognise a huge variety of activating agents (Zheng *et al.*, 2020). NLRP3

inflammasome is the most extensively investigated as it can be activated by a variety of factors, for example, endogenous molecular patterns, like cholesterol crystals, amyloid beta (Aβ) aggregates, and exogenous particles, like silica and carbon nanoparticles, which are used in the industrial world. Therefore, inflammasome activation is extensively studied. Investigation of NLRP3 inflammasome activation in macrophages was mainly carried out using PAMPs, environmental particles and synthetic polymeric nanoparticles (Kelley *et al.*, 2019). However, little research has been done on protein aggregates, especially oligomers of different structure.

Phagocytosed particles can activate the inflammasome via lysosomal destabilisation (Seoane *et al.*, 2020). Cathepsins released from damaged lysosomes mediate NLRP3 inflammasome activation (Campden and Zhang, 2019). Synthetic polymeric particles have been shown to induce cellular response depending on their structural properties (Vaine *et al.*, 2013). In addition, polymeric particles of diverse structure are phagocytosed differently, and the shape of the particle is a decisive factor (Champion and Mitragotri, 2006). This was also demonstrated with Aβ aggregates. Monomers were taken up faster than protofibrils (Gouwens *et al.*, 2016). Another study showed that Aβ fibrils are phagocytosed faster than oligomers and the fibrils up-regulated the phagocytic activity while oligomers attenuated the phagocytosis of fibrils and even fibril-stimulated uptake of fluorescent microspheres in microglia cells (Pan *et al.*, 2011). In general, phagocytosis is a receptor-mediated process, hence, oligomeric proteins of diverse structure can induce different uptake, for instance, α-synuclein fibrils bound to complement receptor 4, which mediated their phagocytosis, while monomers were not detected by this receptor (Juul-Madsen *et al.*, 2020). Factors modulating phagocytic activity also may destine cell activation state. Broader analysis of protein aggregates would answer not only cell biology questions but also show cell activation mechanisms in order to discover the potential treatments for diseases.

Misfolded protein aggregates are found to be one of the inflammation drivers in neurodegenerative disorders (Tejera and Heneka, 2016). Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder all over the world (Zhang *et al.*, 2021b). Although key pathological hallmarks have been identified, the precise mechanisms of disease progression remain unclear to date. It is characterised by the accumulation of Aβ aggregates and the formation of neurofibrillary tau tangles in the brain. Aβ clearance is a crucial process modulating AD progression. Microglia isolated from AD-relevant mouse and human AD patients brain demonstrated Aβ phagocytosis relation to disease severity and aging process (Grubman *et al.*, 2021). Analysis of cell transcriptional profile showed that Aβ plaque-containing microglia had

a dysregulated expression of genes associated with late onset AD. Interestingly, microglia non-containing Aβ had more changes in gene expression which were associated with the accelerated ageing process than microglia with a high Aβ content, thus, being functionally incapable cells. In addition, the phagocytic activity of Aβ plaque-containing microglia was higher compared to isolated microglia cells that did not contain Aβ. These results show the presence of functional diversity of microglia subsets in AD. Furthermore, inflammation, before thought to be a pure bystander, has now been shown to contribute to the AD progression. Aβ itself represents a potent DAMP, which is recognized by receptors present in microglia (Venegas and Heneka, 2017). One of the key players in this process is the NLRP3 inflammasome, which is strongly activated in AD patients and AD-relevant animal models (Venegas and Heneka, 2019). Small Aβ oligomers are neurotoxic and can also be the first direct triggers of inflammation activating immune cells (Mucke and Selkoe, 2012; Walsh and Selkoe, 2020). In addition, neurotoxicity was demonstrated not only with synthetic Aβ preparation but also with natural oligomers of brain tissue applied at nanomolar concentrations, and smaller oligomers were shown as the most important drivers of Aβ toxicity (Yang *et al.*, 2017). Despite the evidence of Aβ cytotoxicity, the studies on initiation of microglia activation by primary pathological Aβ oligomers or other protein aggregates need an extended investigation from mechanistic point of view.

In our study we aimed to investigate macrophage activation by protein oligomers focusing on inflammation, especially on inflammasome activation. Microglia activation by Aβ aggregates was investigated in the context of Alzheimer's disease. A collection of recombinant viral proteins of different structure was selected to represent viral antigens. Inactivated viruses and viral proteins are broadly used for vaccination (Pollard and Bijker, 2021). However, the interaction of viral proteins with innate immune system is barely investigated. Therefore, we explored how they activate innate immune cells – macrophages.

The goal of the study

To investigate macrophage activation by oligomeric proteins of diverse structure – Aβ aggregates and oligomers of viral proteins – and compare their effects.

Objectives

1. To investigate the ability of human Aβ oligomers to induce inflammatory response of macrophages focusing on inflammasome activation.
2. To evaluate the ability of viral oligomeric proteins of different structure to induce inflammatory response of macrophages focusing on inflammasome activation.
3. To identify the mechanism of inflammasome activation by oligomeric proteins.
4. To determine the association between processes related to phagocytosis of oligomeric proteins and inflammasome activation.

Scientific novelty

Our study provides new data on the interaction of innate immune cells interaction with host-associated oligomeric proteins – Aβ aggregates, and pathogen-associated oligomeric proteins – spherical virus-like particles (VLPs) of major capsid protein VP1 of specific human polyomaviruses and filamentous nucleocapsid-like particles of measles and mumps viruses.

The conflicting data exist regarding the ability of Aβ oligomers to induce a pro-inflammatory cytokine response. We demonstrated that NLRP3 inflammasome is activated not only by fibrillar Aβ aggregates as reported before (Halle *et al.*, 2008a), but also by lower molecular weight Aβ aggregates, soluble oligomers and protofibrils. Our results highlight the possibility that microglial activation by these Aβ species may initiate innate immune responses in the central nervous system prior to the onset of Aβ deposition.

Studying viral protein particles, we found that only polyomavirus-derived VLPs mediated inflammatory response in human macrophages. We demonstrated that viral proteins can activate NLRP3 inflammasome depending on their structural properties by a complex mechanism related to the lysosomal damage and K^+ efflux. In addition, we provided new data on the mechanism of inflammasome activation by viral proteins, as this has been widely investigated using polymeric particles and crystals until now (Rashidi *et al.*, 2020). Therefore, we broadened the understanding of how phagocytosed

particles of viral origin activate the inflammasome – an important component of innate immunity. Our findings may explain a mechanism how macrophage and inflammasome are activated by other oligomeric proteins and viral antigens. As polyomavirus VP1 capsid protein is a major viral antigen involved in the virus-host interaction, our approach is based on the use of recombinant VLPs is relevant and demonstrates the impact of viral antigens on the inflammatory responses of innate immune cells.

Our study reveals highly heterogeneous macrophage responses to phagocytosed structurally different oligomeric proteins. Therefore, our findings contribute to a better understanding of mechanisms how macrophages are activated by various antigens. This would benefit the development of vaccines, investigation of immune response to antigens during infection and after vaccination.

Defended statements

1. Aβ oligomers and protofibrils activate NLRP3 inflammasome in the murine microglia.
2. Soluble Aβ aggregates are taken up by microglia independently on the Aβ size.
3. Filamentous oligomeric proteins – nucleocapsid-like structures of measles and mumps viruses – do not induce inflammatory response in macrophages.
4. Spherical oligomeric proteins – polyomavirus VLPs – trigger the inflammatory response followed by NLRP3 inflammasome activation in human macrophages.
5. Polyomavirus-derived VLPs activate the inflammasome by a complex mechanism – via cathepsin release and K+ efflux.
6. Structural properties of oligomeric proteins are one of the factors defining cellular response.

1. LITERATURE OVERVIEW

1.1. Macrophages

1.1.1. Macrophage overview

Macrophages are mononuclear innate immune system cells that respond and remove pathogens and other foreign molecules (Hirayama *et al.*, 2017). These cells reside in different tissues and keep homeostasis together with their defensive function. Macrophages scan their microenvironment and maintain the surrounding by secreting signalling molecules. They recognize foreign molecular structures like pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), which are released after cell damage or other microenvironment disturbance occur (Mosser and Edwards, 2008). These molecules activate macrophages and cause inflammation. It is well known that macrophages participate in inflammatory reactions occurring during pathogen-associated infections or sterile inflammatory diseases (Zwicker *et al.*, 2021).

In general, macrophages are phagocytic immune system cells, defending the organism from the invaders and keeping its homeostasis (Locati *et al.*, 2013). There are two types of macrophages: monocyte-derived and tissue resident macrophages, for example, microglia in the brain, Kuppfer cells in the liver, and osteoclasts in the bone tissue (Fig. 1). First of all, tissue resident macrophages are responsible for the maintenance of the homeostasis to ensure normal tissue function. They scan the surrounding and secret signalling molecules to other cells, remove cellular debris and residues excreted from surrounding cells. Tissue resident macrophages also scan for pathogens and remove them, as well as alert the other cells about danger. These macrophages emerge during the development of organism. They can arise in bone marrow and liver (Davies *et al.*, 2013; Hashimoto *et al.*, 2013) or from the embryonic yolk sack (Ginhoux *et al.*, 2013). For example, microglia cells originated from progenitors in the embryonic yolk sac that seed the brain tissue. After development, tissue resident macrophages persist in their tissue during adulthood. They also can proliferate there depending on the activation signal. Upon infection, peripheral blood circulating monocytes come for help to remove pathogens and restore the tissue (Shi and Pamer, 2011; Locati *et al.*, 2013). Here they become macrophages capable to phagocytose quickly a vast variety of pathogens and other dangerous materials. They also recruit other immune system cells depending on the detected pathogenic molecules. Thus,

monocyte-derived macrophages act in concert with tissue resident macrophages and other immune cells during infection.

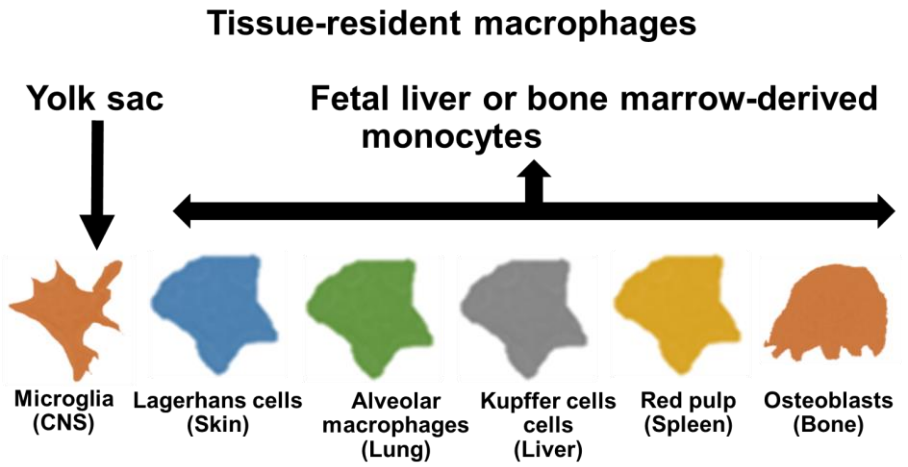

Figure 1. Origin of tissue-resident macrophages: bone marrow-derived monocytes, fetal liver-originated macrophages, and yolk sac-originated macrophages. The image was prepared using information provided at (Pei and Yeo, 2016) and (Italiani and Boraschi, 2015).

1.1.2.Microglia

Microglia are brain macrophages accomplishing defence and regulatory function (Nayak *et al.*, 2014). They are primary immune cells in the brain responding to the pathogens and environmental changes caused by a tissue injury (Fig. 2). Upon activation, microglia cells change their morphology and migrate to the activation site. These cells phagocytose pathogens, remove damaged cells and detect DAMPs. By secreting cytokines and chemokines they recruit other cells to the injury site. Microglia cells are also important for organism development, for example, they participate in the synaptic pruning (Prinz *et al.*, 2019). They also secrete signalling molecules for neurons to maintain their survival and remove metabolites and cellular debris. Microglia play an important role in the development of brain diseases such as neurodegenerative disorders (Dheen *et al.*, 2007). However, whether microglia are good or bad during the progression of disease is unclear. These cells react to environmental changes and announce stress signals. Microglia surrounding Aβ aggregates were clearly seen in the mouse neurodegenerative disease models (Ziegler-Waldkirch and Meyer-Luehmann, 2018) and tissues of Alzheimer's disease patients (Paasila *et al.*, 2020). These cells uptake Aβ plaques and are activated. In addition, microglia activation occurs before the formation of insoluble Aβ plaques (Boza-Serrano *et al.*, 2018). Disability to

degrade Aβ plaques was also demonstrated as damaged lysosomes with Aβ content were detected (Lee and Landreth, 2010). Insoluble Aβ is resistant to lysosomal proteases. Long-lasting cell activation can lead to the microglia malfunction in the context of maintenance of brain homeostasis and induce neuronal death. In addition, Aβ itself is cytotoxic to neurons by inducing dysfunction of synaptic networks (Mucke and Selkoe, 2012).

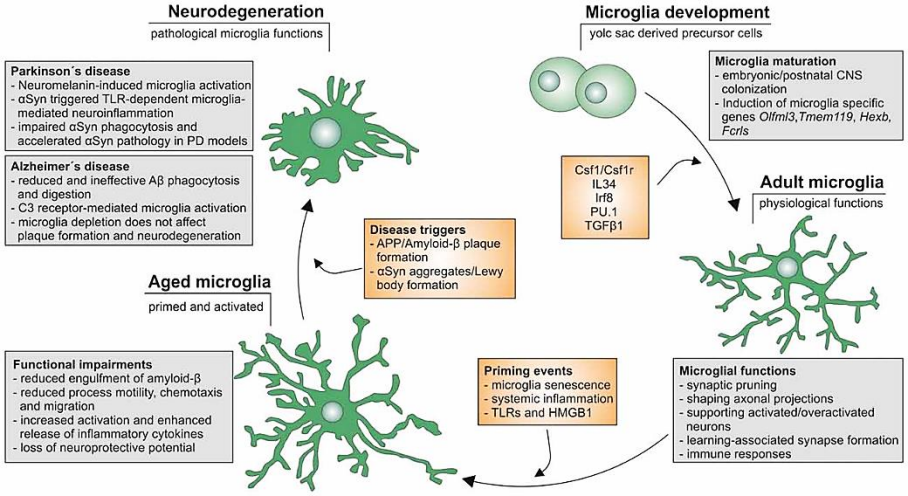

Figure 2. Microglia functions during organism development, adulthood, aging and diseases. Abbreviations: colony stimulating factor 1 (CSF1), interleukin 34 (IL34), interferon regulatory factor 8 (IRF8), transcription factor PU.1, tumour growth factor 1 (TGFβ1), toll-like receptors (TLRs), high mobility group box protein 1 (HMGB1), amyloid precursor protein (APP), α-synuclein (αSyn), Parkinson's disease (PD), central nervous system (CNS), olfactomedin-like 3 gene (*Olfml3*), transmembrane Protein 119 gene (*Tmem119*), hexosaminidase subunit beta gene *(Hexb*), Fc receptor like 2 gene *(Fcrls*). Image was obtained from (Spittau, 2017).

Microglia phenotype around the brain is miscellaneous, for example, it can be disease-associated or ramified (Ochocka and Kaminska, 2021). A new phenotype – disease-associated microglia in neurodegenerative diseases was established (Deczkowska *et al.*, 2018). Alzheimer's disease-associated microglia cells surround Aβ plaques, phagocytose them and secrete inflammatory molecules. In these cells, an increased expression of certain genes is observed. For instance, expression levels of lysosomal, phagocytosis, immune recognition related genes rise up. These cells participate in the removal of protein aggregates, which are resistant to proteases. So, microglia try to prevent pathological process. On the other hand, these cells induce a long-lasting inflammatory response, which has a deleterious effect. In

addition, the exact activation of microglia by certain structures of protein aggregates found during neurodegenerative diseases is unclear. The question is which structures of Aβ – either soluble or insoluble – may activate microglia cells and are the most noxious. It was shown that insoluble Aβ plaques induce inflammatory response in microglia (Navarro *et al.*, 2018), however, cell activation by various structures of soluble Aβ aggregates is less investigated. It is clear that soluble Aβ oligomers induce neuronal damage (Sengupta *et al.*, 2016). However, their effect on microglia is different in a variety of research depending on Aβ structure or impurities of peptides used for preparation of aggregates. On the other hand, Aβ structure is different in the brain of Alzheimer's disease patients and mouse models (Condello *et al.*, 2018). The composition of these aggregates can also be different as Aβ40, Aβ42 and ApoE are found in Aβ plaques (Atwood *et al.*, 2002; Walker, 2020). Therefore, it is important to investigate microglia response to the soluble Aβ aggregates of different structure and size.

1.2. Immunopathogenesis of neurodegenerative disorders, including Alzheimer's disease

AD is the most prevalent neurodegenerative disorder all over the world (Dos Santos Picanco *et al.*, 2018). During AD changes in cytokines, evincing inflammatory or anti-inflammatory body condition, were detected in the blood serum and cerebrospinal fluid (Ray *et al.*, 2007; Rubio-Perez and Morillas-Ruiz, 2012). These changes indicate immune system activation, alteration in immunoregulation and homeostasis. This derangement may occur several years before the onset of clinical symptoms, thus, it could be detected in advance (Ray *et al.*, 2007).

1.2.1. Heterogeneity of microglia and its role in neuroinflammation

Microglia as tissue resident macrophages are very different from peripheral monocyte-derived macrophages. Interesting fact is that microglia cells around the different brain sites are in the different activation states and react in diverse manner depending on its state (Swanson *et al.*, 2020). Microglia activation on site is very local. It was shown that small injures do not cause the spreading of cell signalling and only microglia cells located at injury site are activated. Thus, microglia are different around the brain. This draws attention to disease cases. Phagocytic activity of microglia in the sites of Aβ plaques is comparably lower than that in surrounding cells and Aβ quantity correlates with the rate of microglia phagocytosis (Grubman *et al.*, 2021). Microglia cells located around Aβ plaques are more activated than their

neighbours. This seems a normal effect, however, the inflammatory signal is spreading throughout the brain during the disease and the signal is also transferred to the periphery.

Lysosomal composition is different in microglia cells in healthy and diseased conditions. Dysfunction of lysosomes is related to neurodegenerative diseases and contributes to neuroinflammation (Van Acker *et al.*, 2021). Certain lysosomal components and incapacity to degrade efficiently the cargo can induce cell stress and disturb normal microglia and a neuronal interaction. Changes in lysosomal proteins could be the potential biomarkers of AD (Armstrong *et al.*, 2014) showing lysosomal network involvement in the disease pathogenesis. Moreover, extracellular vesicles play an important role (Fig. 3) – they are used by microglia to transfer components of cellular metabolism, such as enzymes and peptides, for neurons, as well as regulatory and protective molecules (Potolicchio *et al.*, 2005; Lemaire *et al.*, 2019). Via extracellular components cells can also transfer the stress signal and inflammatory signal (Turola *et al.*, 2012; Yang *et al.*, 2018), initiating detrimental processes.

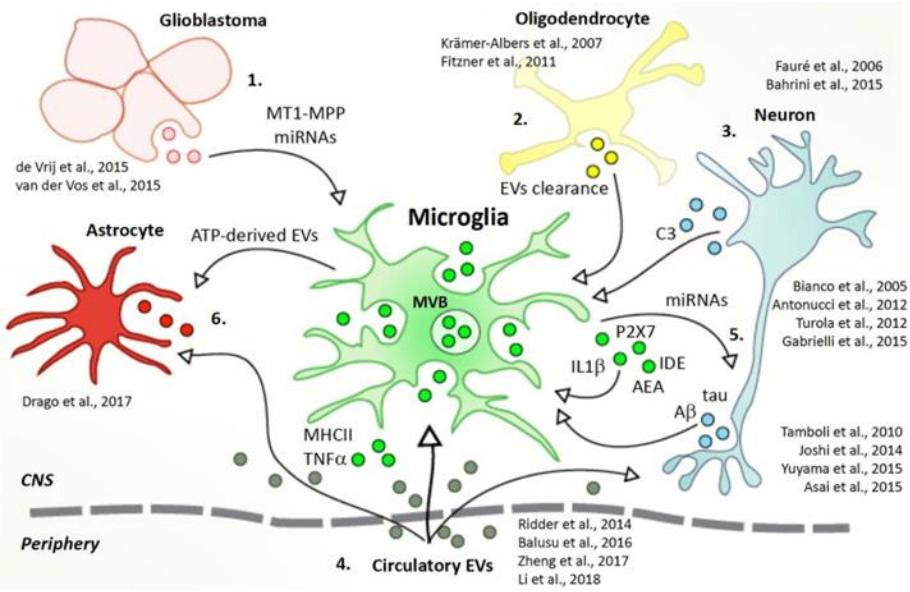

Figure 3. Functions of extracellular vesicles in nerve tissue. The vesicles are transferred in and out microglia making a communication network to maintain homeostasis (Paolicelli *et al.*, 2019).

Microglia cells as tissue resident macrophages are always active by maintaining tissue homeostasis. Even so-called ramified microglia take care of the neuronal function (Prinz *et al.*, 2019). Upon activation, microglia cells

start to proliferate in order to remove the damage faster (Glenn *et al.*, 1992). However, the number of microglia does not change in time. Once the stress factors are removed, the cells tend to acquire the ramified state and multiplied cells die in the apoptotic way. Microglia are long-lived cells (Réu *et al.*, 2017). Proliferation of microglia was detected in neurodegenerative diseases, especially at their beginning (Gómez-Nicola *et al.*, 2013; Gomez-Nicola and Perry, 2016). Cell proliferation is regulated by activation of CSF1 (colony stimulating factor 1, also known as macrophage colony-stimulating factor (M-CSF)) receptor. This molecule is also important for the survival of macrophages and their development from monocytes. Changes in the cell cycle are also related to the Aβ accumulation and development of the neurodegeneration, so, mitosis could be a target for treatment of AD (Rao *et al.*, 2020).

1.2.2. Genetic component of neurodegenerative diseases

Expression of even 832 genes modulates amyloid precursor protein (APP) metabolism and its cleavage (Chapuis *et al.*, 2017). One of the APP cleavage products is the Aβ peptide. It is assumed that 60-80% cases of AD is determined by genetics (Gatz *et al.*, 2006). Interestingly, AD related genes are expressed in microglia cells (McQuade and Blurton-Jones, 2019), highlighting microglia as a promising disease treatment target. The mutations of specific genes and the function disturbance of their coded proteins is related to dementia, such as AD (Bekris *et al.*, 2010). Most of these genes are related to the metabolism of lipids, especially cholesterol, such as the gene encoding apolipoprotein E (ApoE), as well as genes encoding components of innate immunity and endosomal recycling pathway. For example, triggering receptor expressed on myeloid cells 2 (TREM2) acts as a regulating factor and is associated with microglia phagocytic activity and is important for removal of Aβ aggregates (Jiang *et al.*, 2014). In addition, TREM2 protein production increases in aging (Forabosco *et al.*, 2013; Brendel *et al.*, 2017). It acts as a signalling molecule announcing about neuronal death. In addition, the amount of soluble TREM2 correlates with aging and AD biomarkers, such as the load of Aβ42 and phosphorylated tau (Henjum *et al.*, 2016). Changes in the regulation of disease-associated gene expression can initiate dementia. It is assumed that these changes start several years before the disease progression (Guennewig *et al.*, 2021), as well as functional brain changes occur much earlier than the onset of cognitive impairment (Beason-Held *et al.*, 2013). Comparison of healthy individuals and AD patients in gene expression levels

and various brain visualization methods give more information about the development of the disease (Brooks and Mias, 2019).

Single cell RNA sequencing analysis demonstrated the existence of heterogenic microglia populations around the brain (Zheng *et al.*, 2021). Comparing human microglia from healthy and diseased brains, the differences in gene expression are obvious notifying the differences in microglia function and plasticity (Alsema *et al.*, 2020; Olah *et al.*, 2020). In addition, disease-associated microglia was also detected. Epigenetic changes seem to be as important as gene mutations in the development of disease. It was shown that tau pathology can mediate changes in the chromatin organisation (Klein *et al.*, 2019). Chromatin remodelling alters transcriptional profile in neurons and has a detrimental effect. Metabolic abnormalities in AD patients have been monitored by positron emission tomography and these changes were related to the alteration in mitochondrial gene expression (Liang *et al.*, 2008). Disturbance of mitochondrial function can provoke neuronal death. Modulation of microglial gene expression could be a promising tool to treat the disease as these cells transfer the signals to neuronal tissue and may send protection signals, for example, mitochondrial elements or mRNA via exosomes. Induced pluripotent stem cells (iPSCs) used for microglia preparation open the new research area and emphasise the importance of genetic factors in the development of neurodegenerative diseases (Speicher *et al.*, 2019). In addition, the ability to transfer human iPSC-derived microglia into mouse brain presents an incredible tool to study the biology of neurological diseases (Xu *et al.*, 2020). Microglia cells derived from AD patients iPSCs are impaired in phagocytosis, migration, and metabolic activity and this is genetically related (Konttinen *et al.*, 2019). In addition, genetic modifications of iPSC-derived microglia from healthy donors verifies the importance of genetic profile in disease cases. For instance, iPSC-derived microglia with altered *APOE4* expression are not able to phagocytose Aβ as efficiently as wild-type cells (Lin *et al.*, 2018). Cells of AD patients are not only genetically different but also epigenetically, indicating that the disease is already programmed in the entire body, not only in the brain (Yeh and Ikezu, 2019; Penney *et al.*, 2020).

1.2.3. Changes in the microbiota during neurodegenerative disorders and immunoregulation

Gut microbiota is different comparing healthy individuals and AD patients (Vogt *et al.*, 2017; Zhuang *et al.*, 2018; Kowalski and Mulak, 2019), as well as transgenic AD mouse models (Bäuerl *et al.*, 2018). In the patients there are

fewer good bacteria and the bad bacteria promote systemic inflammatory processes. In addition, there is a correlation between the composition of gut microbiota and AD biomarkers. Microbiota secretes metabolites as signalling molecules and in this way may change the entire organism state, promote immunoregulation and modulate host metabolism. In patients with neurodegenerative disease, the disturbance of immunoregulation was described (Chintamen *et al.*, 2020). The decrease in regulatory T cells (Treg) was also detected (Machhi *et al.*, 2020). As it was shown in the mouse model, changes in microbiota composition influence inflammation, Aβ accumulation, microglia activation and cognitive function (Kowalski and Mulak, 2019). Microglia react to systemic organism changes. It was demonstrated that gene expression of microglia dramatically changed after mice got dysbiosis characterised by diminished microbiota (Golomb *et al.*, 2020; Çalışkan *et al.*, 2022). In addition, dysbiosis-induced neuroinflammation and impairment in the cognition were demonstrated (Celorrio *et al.*, 2021). Microbiota has a huge influence on microglia function and liveliness. If dysbiosis occur during the development of organism, microglia morphology and function are changed, even the response to infection is altered and the cells are not able to mature and they start to die (Erny *et al.*, 2015; Lach *et al.*, 2020). Microglia are important for healthy organism and disease development is tightly related to the malfunction of these cells.

Immune system training is one of the ways to change immune response. Investigation of systemic inflammation carried out with mouse models provided an example how immunoregulation and disease development occur. As an example, LPS was injected into mouse blood and cytokines, such as TNF-α, IL-1β, IL-10, were monitored to evaluate the immune training ability (Wendeln *et al.*, 2018). Activation signal in the brain was detected only after the second LPS injection. In the periphery, changes in TNF-α and IL-1β levels were detected after the first injection of LPS and several injections reduced their level but increased IL-10 level in the blood. Long lasting LPS presence diminished the levels of inflammatory cytokines and induced immunotolerance. Microglia were also reprogrammed and LPS-mediated immune training had a positive effect in the AD mouse model by reducing neuroinflammation. Moreover, in another study immune memory induced by repeated low LPS doses protected from sepsis and had a neuroprotective effect, as well as septic condition induced a lower microglia activation (Zhou *et al.*, 2020a). Interestingly, a decrease in Aβ accumulation was observed after LPS injection in the AD mouse model showing that a certain systemic inflammation could modulate neurological disorders (Jendresen *et al.*, 2019). In another study, low doses of LPS also reduced cognitive impairment and tau

pathology in the transgenic mice (Qin *et al.*, 2016). Epigenetic alterations were also identified in microglia after LPS injection resulting in the increased microglia activation (Matt *et al.*, 2016). Moreover, changes in chromatin methylation were detected. In addition, an increase in the production of reactive oxygen species in the brain and cognitive impairment were detected after mouse treatment with LPS (Zhao *et al.*, 2019). Depending on induced inflammation type, immune training or detrimental effect can be caused by inflammatory molecules. Overall, signals from periphery can initiate immunotolerance and reduce the severity of inflammatory diseases.

1.2.4. The interaction of innate and adaptive immunity components in AD

Components of adaptive immunity play a significant role in regulating brain function (Filiano *et al.*, 2015). They communicate with microglia and transfer information from periphery (Fig. 4). It was shown that Tregs modulate microglia function and can supress microglia-induced inflammatory response (Xie *et al.*, 2015). Microglia can also induce formation of Tregs (Ebner *et al.*, 2013). Depletion of Tregs in the AD mouse model caused higher accumulation of Aβ and a cognitive decline while transplantation of Tregs had an opposite effect (Baek *et al.*, 2016). Reduction in Treg number was also detected in AD and multiple sclerosis patients (Ciccocioppo *et al.*, 2019). Therefore, despite the fact that adaptive immunity cells do not reside in the brain, their regulatory function is obvious. Cells from periphery can enter the brain via CCR7 (Noor and Wilson, 2012). In the zone of meningeal lymphatic vessels, T cells enter the brain depending on the brain and periphery signalling. Then, these cells can influence brain function directly. In a mouse model, Th1 cells enrolled into the brain promoted progression of AD pathology (Browne *et al.*, 2013). T cells interaction with microglia stimulated inflammatory response. Therefore, adaptive immunity contribution to the development of neurodegenerative disorders is considerably high.

T cell role was also demonstrated in AD immunotherapy (Monsonego *et al.*, 2013). Super high activation of these cells was detected after patients' immunisation with Aβ vaccine. The progression of the disease instead of improvement was induced, although Aβ load was reduced (Panza *et al.*, 2019). Despite the fact that side effects are individual, evidence of T cell role in the brain immunity is emphasised. Interesting fact is that for one person the immunotherapy works and for others it does not. It could be that microglia cells are not the main players inducing inflammation in the brain. Their activation could be beneficial in neuroinflammatory process maintained or caused by adaptive immunity cells. In addition, immunotherapy could show

beneficial effect before disease progression and may be used as a preventive strategy (Overk and Masliah, 2019), like previously mentioned LPS-induced immune training in AD mouse models.

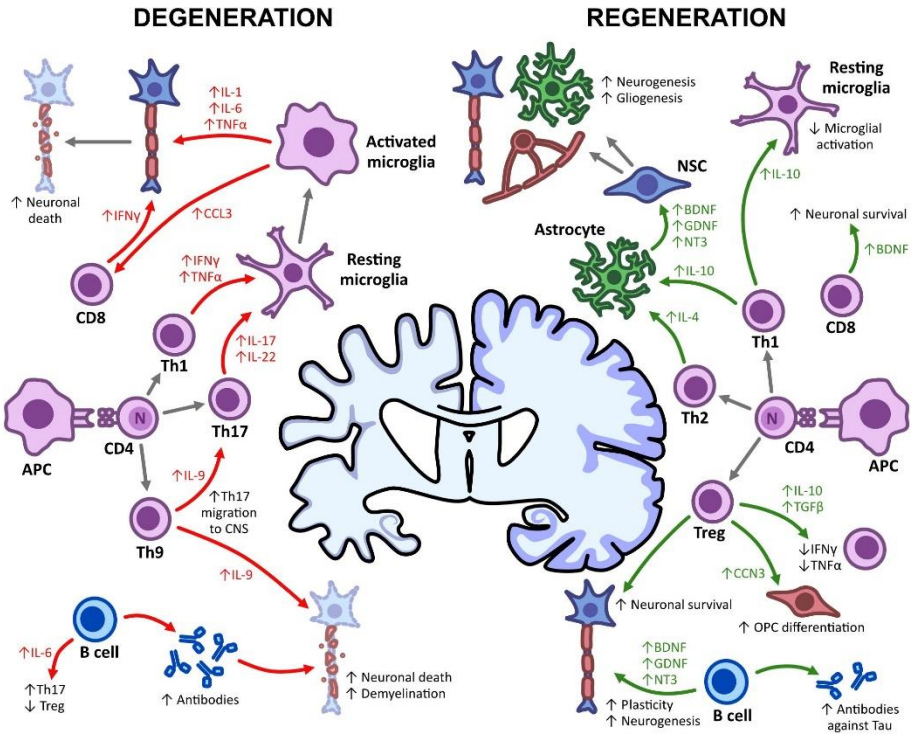

Figure 4. Innate and adaptive immunity interaction in neurodegenerative disorders and possible mechanisms of regeneration. Image was obtained from (Mayne *et al.*, 2020).

Despite the fact that inflammation is kept as a driver of dementia, anti-inflammatory components can also be deleterious. For instance, IL-10 had a negative impact on Aβ phagocytosis and induced its accumulation and cognitive decline in the APP transgenic mice (Chakrabarty *et al.*, 2015). IL-10 induced the binding of ApoE to Aβ aggregates and disturbed their uptake. IL-10 negative effect on amyloidosis was also demonstrated in another study. Deficiency in IL-10 enhanced Aβ clearance and restored cognitive decline in APP/PS1 mice (Guillot-Sestier *et al.*, 2015). In addition, another study showed that IL-10 deficiency promotes neuroinflammation and tau pathology (Weston *et al.*, 2021). However, analysis of frontal cortex tissue material of AD patients showed that IL-10 is not associated with tau pathology or other AD hallmarks (Friedberg *et al.*, 2020) demonstrating the importance of immunoregulation in neurodegenerative diseases.

1.2.5. Immunotherapy of Alzheimer's disease

The removal of Aβ aggregates by immunotherapy is a complex process. Despite the decrease in Aβ accumulation after immunotherapy, the cognitive function did not improve in most cases (Panza *et al.*, 2019). It was demonstrated that antibodies are not sufficient in the process of Aβ clearance. For example, the use of TLR agonists induced microglia activation and improved Aβ removal and cognition in the mouse model (Michaud *et al.*, 2013). It is assumed that TLR4 could be a target in AD treatment due to the relation between TLR gene polymorphism and AD risk (Zhou *et al.*, 2020b). Phagocytic activity of microglia in the sites of Aβ plaques is comparably lower than in surrounding cells (Grubman *et al.*, 2021). Additional activation of phagocytic cells could improve Aβ clearance. Co-culturing aged microglia from AD mouse with young microglia from wild-type mouse in organotypic brain mediated aged microglia proliferation and clustering around Aβ plaques inducing their clearance (Daria *et al.*, 2017). In addition, granulocyte macrophage colony stimulating factor (GM-CSF) had the same effect as young microglia showing the plasticity of even aged microglia.

1.2.6. Brain sensitivity and aging related processes

Our brain cells are sensitive and sometimes not adapted to cope with damage. Any changes in brain environment set in motion microglia cells. For example, after brain trauma, especially severe sport-related injuries, changes in microglia are detected (Izzy *et al.*, 2019). Injuries change microglia gene expression profile resulting in reduced ability to maintain homeostasis. Another outcome is alteration of neuronal function resulting in damaged nervous tissue (Carron *et al.*, 2016). Their physiological processes become disturbed and they start to accumulate tau protein. Normally tau protein is associated with microtubule and maintain its stability (Barbier *et al.*, 2019). After traumatic brain injury, tau aggregates are accumulated inside and outside neurons (Edwards *et al.*, 2020). Once microglia cells detect tau proteins, they start to phagocytose and degrade them (Vogels *et al.*, 2019). However, the inability to break down insoluble tau aggregates and at the same time to maintain the homeostasis cause even higher neuronal damage. On the other hand, if microglia are busy with cleaning of the surrounding aggregates they are not able to maintain neuronal function by secreting signalling molecules and removing by-products. Only keeping our brain calm can ensure their proper work. For example, time lapse experiments of AD in mouse model demonstrated that in the early state microglia made beneficial function (Baik *et al.*, 2019). However, once the pathological conditions progressed, the cells

were not able to cope with DAMPs, they even got deficit in energy metabolism and became metabolically defective cells, which were unable to react properly to damaged sites. Microglia are very important for limiting Aβ plaque formation (Zhao *et al.*, 2017), thus, malfunctioning microglia cells are responsible for pathological processes in the brain.

Neuroinflammation is linked to microglia activation and positron emission tomography technique allows to monitor cell activation and at the same time disease development *in vivo*. Using APP/PS1 mouse model it was demonstrated that positron emission tomography imaging perfectly correlates with pathological conditions and microglia activation (Hu *et al.*, 2020), thus, animal studies could be used to find out the appropriate treatment at a certain time of disease. In another mouse model, taupathy mice, characterised by phosphorylation and accumulation of extracellular tau, microglial activation was linked to the pathological tau accumulation and brain atrophy (Sahara *et al.*, 2018). In addition, positron emission tomography imaging analysis showed different microglia activation during different stages of neuropathological condition development (Beaino *et al.*, 2021), though, microglia activation is increased in both early and late AD stages (Cagnin *et al.*, 2001; Versijpt *et al.*, 2003; Okello *et al.*, 2009; Femminella *et al.*, 2019), as well as in Parkinson's disease patients (Iannaccone *et al.*, 2013). This highlights the presence of microglia changes at the beginning of neurodegenerative diseases.

Our brain in general is not adapted to aging process. Therefore, microglia cells also do not adapt quickly to changes associated with the senescence and can be chronically activated causing damage to surrounding tissue or become dystrophic cells (Greenwood and Brown, 2021). In aging the activation and proliferation of microglia increase, however, they can also become malfunctioning cells. Senescent microglia have increased production of complement proteins and other inflammatory molecules (Schartz and Tenner, 2020). During inflammatory reaction, microglia can ingest damaged neurons instead of helping to restore homeostasis or kill them by secreting DAMPs (Brown and Neher, 2010). Thus, interventions regulating inflammatory response and contributing to the maintenance of homeostasis are promising tools to combat with neurodegenerative diseases, like AD.

1.3. Macrophage activation

1.3.1. Macrophage activation pathways

Macrophages have an immense variety of receptors on their surface. Upon activation, certain signalling pathway is triggered. Macrophages can be activated to these major states: inflammatory (classically-activated), anti-inflammatory, damage repair or regulatory state (Fig. 5). The latter states are classical macrophage activation states. However, especially in disease conditions these immune cells can be in the several activation states.

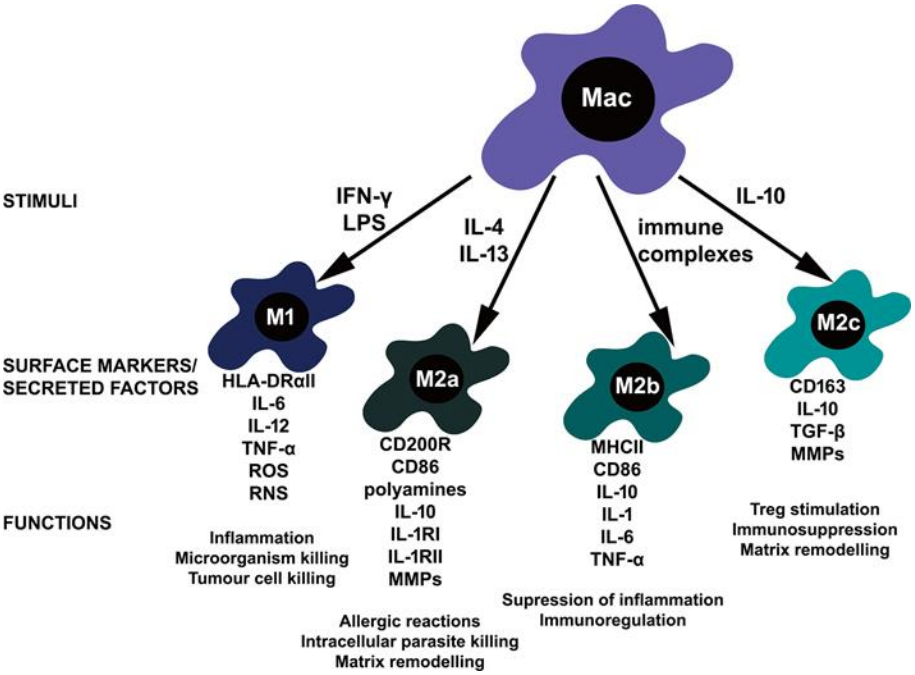

Figure 5. The Schematic representation of macrophage polarisation. Image was obtained from (Sousa and Määttä, 2016).

In detail, macrophages could be grouped according to the classical activation states: inflammatory (M1) and anti-inflammatory (M2) macrophages (Mosser, 2003; Mantovani *et al.*, 2004; Gordon and Taylor, 2005; Edwards *et al.*, 2006; Mosser and Edwards, 2008). M2 are categorised into more subpopulations: M2a, M2b and M2c. M1 macrophages promote inflammation by secreting various cytokines and chemokines to recruit other immune cells. In most cases this subpopulation is formed after encountering a pathogen. M1 macrophages phagocytose pathogens and promote other immune cells, like neutrophils, to destroy them. M1 macrophages are characterised by several inflammatory markers, such as secretion of IL-12, IL-

23, TNF-α, IL-1, IL-6, reactive oxygen species, expression of NO synthase, MHCI, CD86 and Fc receptors exposure on their surface. These cells kill intracellular pathogens and initiate development of Th1 cells. They also detect opsonised pathogens, such as coated by antibodies or complement proteins.

M2a macrophages secrete anti-inflammatory mediators, for example, IL-10, IL-1ra, IL-1RII and even small level of inflammatory molecule IL-12. They promote the development of Th2 cells and participate in allergic reactions. These cells are also responsible for removal of nematodes/parasites. In addition, M2a subpopulation is characterised by increased expression of MHCII, mannose receptors and arginase synthesis. M2b macrophages activate Th2 cells and participate in the development of immunotolerance. They are characterised by release of IL-10, TNF-α, IL-1β, IL-6, and small amounts of IL-12. M2b cells also express higher levels of CD86 and mannose receptors. M2b subpopulation is generated after an encounter with immune complexes composed of antigen and antibody and it contributes to the humoral immunity (Anderson and Mosser, 2002; Mantovani *et al.*, 2004). M2c macrophages secrete IL-10 and TGF-β. These cells are also characterised by the high expression of mannose receptor and low expression of MHCII. M2c macrophages are anti-inflammatory and immunoregulatory cells. They are responsible for the termination of inflammatory reaction and regulation of inflammation. They also secrete molecules stimulating the restoration of damaged tissue.

One macrophage subpopulation regulates other subpopulation function in order to maintain heathy tissue. For instance, IL-4 and IL-13 secreted by M2 macrophages induce expression of IL-1ra which is an antagonist of IL-1 receptor (Mantovani *et al.*, 2004; Mosser and Edwards, 2008). However, especially in disease cases, the classical macrophage subpopulations do not exist. In other words, these populations are intertwined and simultaneously express M1 and M2 marker. Disease-associated macrophages exist in chronic inflammation. These cells are hyperactivated and incapable to ramify due to permanent activation. For example, microglia surrounded by Aβ plaques put efforts to remove these protein aggregates, however, due to inability to degrade them, stay in hyperactivated state secreting inflammatory and anti-inflammatory molecules at the same time. In addition, microglia cells are in multiple phenotypes and they can have neuroprotective or neurotoxic properties (Sarlus and Heneka, 2017). Therefore, in disease cases the best option is not to look for one macrophage subpopulation but detect as many markers as possible to identify macrophage state (Ginhoux *et al.*, 2016). In addition, macrophages are plastic cells so they can turn from one activation

state to another (Okabe and Medzhitov, 2016). However, the disbalance in immunoregulation leads to the unrestrained inflammatory response.

1.3.2. Inflammatory molecules secreted by macrophages

Macrophages secrete a variety of chemokines and cytokines (Mantovani *et al.*, 2004; Murray and Stow, 2014). Chemokines are responsible for the recruitment of other immune cells to the infection site. Hence, their secretion is related to inflammatory processes, however, certain chemokines play a homeostatic function. Cytokines signal about danger, such as tissue damage and pathogens, or microenvironmental changes. They regulate various biological processes including immune reactions. They can be classified to inflammatory and anti-inflammatory molecules (Dinarello, 2007). Cytokines transfer signals to the cell and induce their proliferation, apoptosis, differentiation, ability to secrete anti-microbial molecules or cytokines to other immune cells in order to enhance immune reaction. Other cytokines are responsible for immunoregulation and termination of immune response. Impairment of regulation of these cytokines may cause immune disorders and provoke pathological processes leading to the tissue damage (Arango Duque and Descoteaux, 2014).

One of well-known inflammatory cytokines is tumour necrosis factor (TNF-α) (Aggarwal *et al.*, 2012). This cytokine is secreted not only by macrophages but also by dendritic cells, lymphocytes, NK cells, neutrophils and some non-immune cells. TNF-α activates various immune cells by provoking inflammatory reactions via NF-κB pathway activation. Announcing about pathogens TNF-α initiate their removal by other immune cells. This cytokine also initiates not only expression of inflammatory molecules (such as IL-1β, IL-6, IL-8, IL-18, chemokines, nitric oxide synthase) but also apoptosis and cell proliferation depending on its concentration and receptors to which it binds. Normally TNF-α promotes apoptosis to damaged and abnormal cells, for example cancer cells or virus-infected cells. Other cytokine IL-1β is well-known as a trigger of strong inflammatory reactions during infection (Dinarello, 2018). IL-6 is also very important in the inflammatory processes (Tanaka *et al.*, 2014). It initiates illness symptoms like fever via induction of prostaglandins production. Anti-inflammatory cytokine IL-10 is important for immunoregulation and termination of inflammation (Ouyang and O'Garra, 2019). It also induces tissue repair. Overall, cytokines are critical for healthy tissue, however, pathological conditions may provoke their dysregulation leading to diseases. The changes in cytokine levels could notify about cellular function

disturbance, immune system activation and the development of pathological process (Vilcek and Feldmann, 2004). For example, an increase in TNF-α shows inflammatory process due to pathogens, metabolic disorders, autoimmune or neurological diseases, including AD (Aggarwal *et al.*, 2012) (Swardfager *et al.*, 2010).

1.3.3. Macrophage receptors

Professional phagocytes, macrophages expose many receptors on their surface to detect changes to the environment and especially pathogenic molecular patterns (Taylor *et al.*, 2005). A lot of intracellular receptors are also expressed by macrophages for further recognition of endocytosed particles or detection of invaded pathogens, like viruses. Innate immunity receptors are called pattern recognition receptors (PRR). They recognise pathogen-associated molecular patterns (PAMP) or damage-associated molecular pattern (DAMP). Examples of PAMPs and DAMPs and their receptors are presented in Table 1 (Mogensen, 2009; Gong *et al.*, 2020). Most of the receptor families, which recognise PAMPs, also recognise DAMPs.

Table 1. Examples of PAMPs and DAMPs and their receptors

Molecular pattern	**Examples of PAMPs and DAMPs**	**Receptors**
PAMP – molecules of pathogens	Bacterial molecules, like lipopolysaccharide (LPS), bacterial lipoproteins, peptidoglycan, lipoteichoic acids, mannose-rich glycans, flagellin; viral nucleic acid, like double-stranded or single-stranded viral RNA; glucan zymosan found in yeast cell wall, etc.	Toll-like receptors (TLR), from TLR1 to TLR10; NOD-like receptors (NLR), like NLRP3; RIG-I-like receptors (RLR), for example, retinoic acid inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5); DNA sensors, like AIM2; scavenger receptor (SR), for example, SR class A (SRA) or class B (SRB).

DAMP – molecules of danger, generated in organism	Heat shock proteins, hyaluronic acid, altered membrane phospholipids; molecules generally found inside the cells, like ATP and endogenic DNA; molecules normally located inside lysosomes that enter the cytosol after membrane damage, for example, cathepsins and immune complexes having microbes; ubiquitous nuclear protein, called high mobility group box protein 1 (HMGB1), S100 – calcium binding proteins	C-type lectin receptors, for example, macrophage-inducible C-type lectin (MINCLE), dectin-1, which recognise N-glycans, dendritic cell natural killer lectin group receptor 1 (DNGR1); RLR; NLR; receptor for advanced glycation products (RAGE); triggering receptors expressed on myeloid cells (TREM), for example, TREM1, which recognise HMGB1 and heat shock proteins, and TREM2, which ligands are lipid components and lipoproteins, like phosphatidylserine and phosphatidic acid, APOE apolipoprotein, low- and high-density lipoproteins; TLR, for example TLR2 and TLR4 recognise HMGB1; G-protein-coupled receptor (GPCR), for example, which senses Ca2+ changes, like GPRC6A, P2Y2 receptor, which ligand is ATP, N-formyl peptide receptor 1 (FPR1), which senses cathepsin G, and FPR2, which detect Aβ42, oxidised low-density lipoproteins, serum amyloid A and other peptides; ion channels, for example, P2X7 receptor, which senses ATP, and transient receptor potential ion channel, like, transient receptor potential cation channel subfamily M member 2 (TRPM2), which detects reactive oxygen species.

After recognition of pathogenic or danger-associated molecules by PRR, an immune response is induced (Murray and Wynn, 2011). Strong

activators, like LPS, cause strong inflammatory response. Pathogenic molecules are classical immune system activators, however, sterile inflammation is becoming a huge problem nowadays (Gong *et al.*, 2020). Our lifestyle is changing and new inflammatory diseases are diagnosed. Therefore, a new concept lifestyle-associated molecular patterns (LAMPs) was established (Zindel and Kubes, 2020). LAMPs seem to be different from other sterile inflammation-associated molecules, DAMPs, and are indicated as factors causing lifestyle-associated inflammatory diseases. Examples of LAMPS can be endogenous molecules, like cholesterol crystals, monosodium urate crystals, oxidized lipoproteins, calcium pyrophosphate dihydrate, and exogenous particles, like silica and asbestos. Our immune system defends us from pathogenic compounds by phagocytosis and the inflammatory response (Murray and Wynn, 2011).

1.3.4. Inflammation and the role of macrophages in inflammatory reactions

Inflammation is described as immune response to certain agents causing the secretion of inflammatory cytokines and other signalling molecules in order to notify other cells about infection or tissue damage (Chen *et al.*, 2018). Inflammation can be induced by infectious and environmental factors, such as pathogens, damaged cells, DAMPs and noxious materials. These agents activate immune cells and trigger inflammatory response. Various immune cells like monocytes, macrophages, neutrophils, are recruited to the damage site. These cells secrete antimicrobial molecules, phagocytose pathogens, secrete signalling molecules to report about damage type and recruit other cells. Macrophages as professional phagocytes remove pathogens, cellular debris and secrete growth factors and cytokines to notify other immune cells like lymphocytes or repair the damage tissue (Fujiwara and Kobayashi, 2005). These phagocytes have a huge variety of intracellular and extracellular receptors, therefore, they can respond to the vast agents in a certain way (Taylor *et al.*, 2005). As an example, TLR4 recognises bacterial LPS and in the meantime viral RNA is recognised by the intracellular TLR3. Bacteria and viruses induce different cytokine secretion to activate distinct cell types. Tissue resident macrophages are present in certain tissues all the time. Thus, they are one of the first cells responding to microenvironmental changes. Macrophages detect pathogens and other particles very fast in order to ensure quick elimination of possible damage. In the cases where are a huge damage or plenty of pathogens, continuous cell activation can cause chronic inflammation and damage the surrounding tissues (Parisi *et al.*, 2018). In

addition, macrophages could drive inflammation in sterile inflammatory diseases, such as type 2 diabetes, atherosclerosis, and periodontitis.

1.4. Endocytosis

1.4.1. Endocytosis pathways

Macrophages scan their surroundings and detect various compounds using cell surface receptors (Taylor *et al.*, 2005). Surrounding materials also can be ingested and detected by intracellular receptors. The compounds from the environment enter the cells either by diffusion or active process called endocytosis (Miaczynska and Stenmark, 2008). During this process the compounds are engulfed by the cells after formation of membrane-bound vesicle. Vesicle formation is mediated by plasma membrane rearrangements and actin cytoskeleton remodelling. Endocytosis is divided into several types – macropinocytosis, phagocytosis and micropinocytosis (Fig. 6) (Doherty and McMahon, 2009). The endocytosis pathway is determined by material structure, size and interaction with specific cellular receptors. Macropinocytosis is mainly the pathway to soak up the nutrition and fluids. The size of cargo is less than 500 nm. The ingested material is fused with lysosome for degradation. However, sometimes microorganisms and their elements bind to the cell surface non-specifically and are ingested by the cell. Therefore, not only nutrition materials but also some pathogens get inside the cells via macropinocytosis (Underhill and Goodridge, 2012).

Phagocytosis is a specific endocytosis and usually it is related to immune cells, such us macrophages, dendritic cells and neutrophils, which internalise large particles (>500 nm), like pathogens, cellular debris and apoptotic cells (Uribe-Querol and Rosales, 2020). However, smaller particles can also bind to phagocytic receptors and be internalised by this pathway. These particles can be misfolded protein aggregates. This type of endocytosis is normally a defensive reaction against infection and foreign substances in the body. Phagocytosis is based on receptor-ligand interaction leading to the activation of certain cell signalling pathway. Cell surface receptors of phagocytes are divided into opsonic and non-opsonic receptors. Non-opsonic receptors recognise molecules to be ingested directly while opsonic receptors recognise molecules coated by host proteins. The examples of non-opsonic receptors: C-type lectins, like Dectin-1, Dectin-2, Mincle, and DC-SIGN; lectin-like recognition molecules, like CD33; and scavenger receptors. There are several pattern recognition receptors, such as toll-like receptors (TLRs), which can enhance the phagocytosis of the particles (Iwasaki and Medzhitov, 2015).

TLRs recognise surface patterns of pathogens or their genetic material (Kawasaki and Kawai, 2014). The examples of opsonic receptors are Fc receptors (Goodridge *et al.*, 2012; Tay *et al.*, 2019) and complement receptors (van Lookeren Campagne *et al.*, 2007). Virus clearance by antibody-dependent cellular phagocytosis is a classic example of Fc receptors activity while antibody coated viruses are internalised by phagocytes (Tay *et al.*, 2019). Next to antibodies and complement components the opsonins can be fibronectin, vitronectin and mannose-binding lectin (Flannagan *et al.*, 2012). These molecules serve as a target for phagocytes.

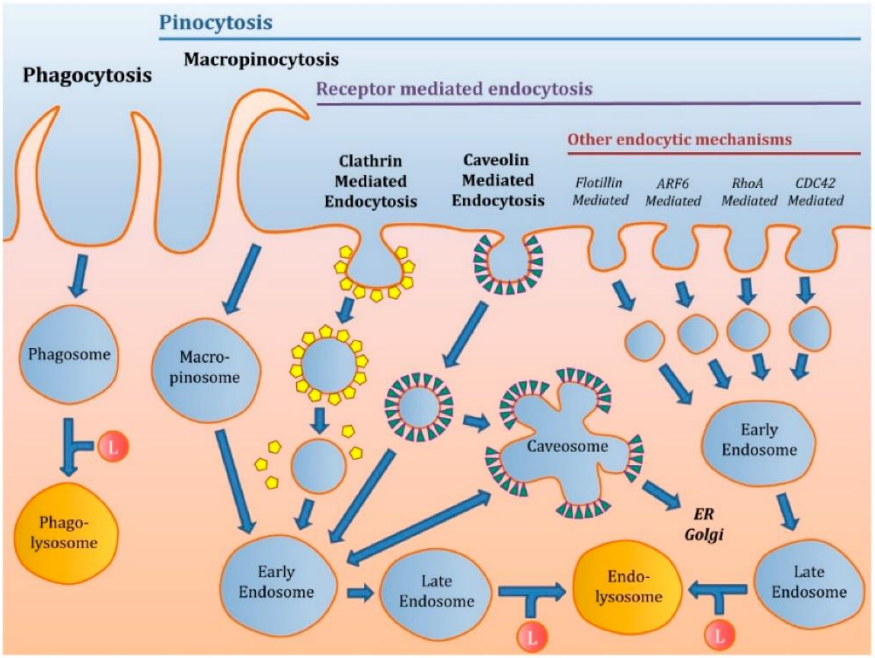

Figure 6. The endocytosis pathways. The internalized vesicles fuse with early endosomes in which the sorting begins. Depending on the pathway and cargo content the vesicles can be sorted and recycled back to the plasma membrane, sent to the Golgi network via retrograde trafficking mechanisms or trafficked to the lysosome for degradation, like phagosomes. Abbreviations: Rho-GTPases – RhoA, CDC42; ER – endoplasmic reticulum; ARF6 – ADP-ribosylation factor 6 (GTPase). Image was obtained from (Manzanares and Ceña, 2020).

Phagocytosis as other endocytosis processes comprises of several steps: particle recognition mediated by cellular receptor and phagocytosed material interaction leading to cell signalling; particle ingestion during actin cytoskeleton remodelling; phagosome formation; phagosome fusion with

lysosome – phagolysosome formation; cargo degradation and activation of intracellular receptors (Rosales and Uribe-Querol, 2017). Phagocytosis is mostly mediated by receptors (Freeman and Grinstein, 2014). Pathogens and other particles are internalised via phagocytosis and depending on phagocytosed agent and the surrounding cells induce further activation. After particle binding to receptors, specific sequence of cell signalling cascade is initiated. Only certain structure and size particles can activate further processes in phagocytes. Phagocytosis of apoptotic or damaged cells is also an important macrophage function. The uptake of both pathogens and damaged or pathogenic cells initiates macrophage activation. Thus, phagocytic activity is important for maintenance of tissue homeostasis (Hirayama *et al.*, 2017).

Macrophages phagocytose not only pathogens and pathological materials but also dead or cancerous cells (Zhou *et al.*, 2021). After engulfment of tumour cells macrophages present tumour-specific antigens for adaptive immunity cells to induce antitumor activity. The dead cell surface notifies about death pathway and macrophage activation depends on the engulfed content telling about existing danger. When the cells dye in the apoptotic way, there are no infection signs. Macrophages detect these cells via phosphatidylserine and no inflammatory response is induced (Torchinsky *et al.*, 2010). Macrophages secrete anti-inflammatory molecules, like IL-10, however, if the encounter with TLR agonist occurs, inflammatory cytokines, like IL-6, also are secreted leading to Th17 development. Th17 is important for protection from infection and restoration of damage tissues (Torchinsky *et al.*, 2010). Meanwhile, when the cells dye in the necrotic pathway due to infection or tissue damage, phagocytes also get danger-associated signal (DAMPs) leading to macrophage activation. DAMPs, such as heat shock proteins and genetic material, inform about the stress. Depending on their type, different cellular response is generated (Green *et al.*, 2009). Therefore, necrosis outcome could be a strong inflammatory response, like PAMP-induced cell activation, when inflammatory molecules are produced (Blander and Medzhitov, 2006).

Micropinocytosis, which is responsible for the internalisation of small particles (<200 nm), has several heterogeneous pathways: clathrin-mediated endocytosis, caveolin-mediated endocytosis, flotillin-mediated endocytosis, fast endophilin–mediated endocytosis, and others, such as clathrin-independent carrier/GPI-anchored protein enriched early endosomal compartment (CLIC/GEEC) pathway (Kumari *et al.*, 2010). Micropinocytosis in general is a receptor-mediated endocytosis pathway which are subdivided

on the basis of certain features. The main characteristics of these pathways are the presence of coated proteins (clathrin and caveolin) and the dependence on dynamin. As micropinocytosis is receptor-mediated, the outcome of internalised particle is fated by the different signalling pathway depending on the specific receptor bound to a ligand (Sorkin and von Zastrow, 2009).

During caveolae-mediated endocytosis the particles are trafficked to bulb-shaped caveolae invaginations on the cell membrane following internalisation of the particle (Kiss, 2012). This endocytosis pathway is triggered by ligand-receptor interaction, thus, it is particularly regulated and specific substances are taken up by this pathway. Caveolae mediates the internalization of a variety of agents, including sphingolipids and its binding compounds, glycosylphosphatidylinositol (GPI)-anchored proteins, endothelin, growth hormones, viruses (for example, SV40), and bacteria (Nabi and Le, 2003).

During CLIC/GEEC pathway, uncoated tubular vesicles are formed from plasma membrane (Kalia *et al.*, 2006). Then this vesicle matures into early endocytic compartments called GPI-anchored protein enriched compartments. GEEC is then fused with sorting endosomes in a Rab5 and PI3K dependent manner. In CLIC/GEEC pathway depending on the cell type early endosomes can be directed to lysosomes or to the pericentriolar recycling compartments. This tubulovesicular carries are different from clathrin- or caveolin-mediated pathways during which spherical vesicles are formed. During this clathrin-independent process a large volume of fluids is ingested at the same time (Conte and Sigismund, 2016).

Clathrin-mediated endocytosis is characterised by the formation of clathrin-coated pit after clustering of the endocytic proteins at the plasma membrane (Picco and Kaksonen, 2018). Coated vesicle is separated from membrane and later uncoated. The size of endosome is about 100 nm (Blander and Medzhitov, 2006; Doherty and McMahon, 2009). Formed endosome participate in further endosomal trafficking processes depending on its cargo. A wide-range of materials are transferred from cell exterior to their interior via clathrin-mediated endocytosis. The cargo can be nutrition or cell signalling molecules. They are important for cell physiological function and regulate its development, cell adhesion, inform the cell about its surroundings (Kaksonen and Roux, 2018). Therefore, receptor dependant clathrin-mediated endocytosis is important for the physiological cell function while phagocytosis is crucial to fight with pathogens.

1.4.2. Macrophage response to particles of various size and structure

Macrophages respond to activation agents depending on its structural properties and size. Research data on nanoparticles show that macrophages engulf particles of distinct structure in a different way (Richards and Endres, 2016). These particles can be taken up by clathrin-mediated endocytosis, receptor-mediated phagocytosis, caveolae-mediated endocytosis or pinocytosis (non-specific extracellular fluid uptake). Chemical and physical properties of the particles predestine the type of cell activation pathway (Sabourian *et al.*, 2020). Depending on the type of endocytic pathway the engulfed particles induce inflammatory, anti-inflammatory or neutral response in macrophages (Baranov *et al.*, 2020).

Macrophages can detect viruses by one of the endocytosis pathway, called macropinocytosis. Solid particles or fluids enter the cells via this pathway after formation of large endosome (Falcone *et al.*, 2006; Kerr and Teasdale, 2009). Macropinocytosis is initiated by the activation of receptor tyrosine kinases. Then, cell signalling cascade activates cell movement and endocytosis. Micropinocytosis is also related to the antigen presentation on the cell surface. Certain viruses, for example, vaccinia enter the host cell by micropinocytosis. Viruses have phosphatidylserine residues, which is found on apoptotic cells – ligand for apoptotic cell internalisation (Mercer and Helenius, 2009). Therefore, viruses can enter host cells via apoptotic cells endocytosis pathway.

Despite many studies on the effect of particle structure on its phagocytosis, the unanswered question is how phagocytosed particle size determines macrophage immune response. Particle structure can predestine cell activation pathway as it was shown that activating agent size, shape, and even surface roughness are important parameters for their phagocytosis and subsequent immune responses, especially inflammasome activation (Baranov *et al.*, 2020). Particles having rough surface and a lot of spikes are the most prone to induce inflammasome activation and cell death while smooth particles do not. Another study demonstrated that rod-shape particles of bacteria size (2-3 μm) were phagocytosed by murine macrophages more efficiently than spherical particles emphasising that our immune system rapidly reacts to agents mimicking bacteria shape (Doshi and Mitragotri, 2010). They also showed that larger particles are ingested faster, thus, should induce different cell activation as presented in the study, investigating particles of RNA and protamines (arginine-rich nuclear proteins). Nano-sized particles (represent viruses) induced secretion of INF-α and micro-sized particles (represent bacteria) – release of TNF-α (Rettig *et al.*, 2010). Therefore, size dependent immune response is driven by cellular receptors as interferons are strongly

secreted after viral infection. It was also demonstrated that rod-shape particles from poly(methacrylic acid) polymer induced stronger inflammatory response than spherical particles, although they were phagocytosed in a similar way by human monocyte-derived macrophage (Chen *et al.*, 2016). Other study showed that 1 μm polystyrene particles induced inflammatory cytokine secretion comparing to nano-sized (0.078 μm) particles (Rothen-Rutishauser *et al.*, 2007). Macrophages as innate immune cells are known to respond to bacteria. Another study also endorsed that bacteria shape (rod-shaped) particles induce inflammatory responses contrary to spheres independent on particle material (Garapaty and Champion, 2019). In addition, study on immune response to structurally different hydroxyapatite particles, ranging 0.1-100 μm in size and needle-shaped or spherical form, showed that needle-shaped and smaller particles are the most prominent inducers of pro-inflammatory cytokine IL-1β (Lebre *et al.*, 2017). The latter data reveal the evolutionally developed immune response of phagocytes emphasising the importance of phagocytosed material size.

The studies on host response to various endogenic materials are important to understand the response of immune cells, including macrophages, to nano- and micro-particles as various organic and inorganic materials are used in implantation and host response inert materials are prioritised (Saini *et al.*, 2015). In addition, having broader understanding how to modulate immune response, biomaterials that can dictate a favourable macrophage response after implantation are being developed (Li *et al.*, 2016). Possibility to avoid the negative host immune response allows a successful implant integration into target tissue since implant composition can have a plausible effect on the inflammatory response.

1.5. The inflammasome

1.5.1. Overview of inflammasomes

Inflammasomes are innate immune system receptors recognising conservative microbe patterns (PAMPs) and molecules evincing tissue damage (DAMPs) (Kanneganti, 2015). The activation of inflammasome occurs in several steps and each of them are tightly regulated. The result of inflammasome activation is the release of cytokines and alarmins that enhance immune response, and pyroptosis that abolishes the places for pathogen replication. During evolution process, host-pathogen interaction fated bacterial and viral pathogens to develop a range of molecular inhibitors

targeting each step of inflammasome activation to avoid host response (Garib *et al.*, 2016).

The inflammasome is formed of multimeric protein complex after response to the activation signal (Broz and Dixit, 2016). They are composed of NOD-like protein receptors (NLR) or other proteins depending on the inflammasome, apoptosis-associated speck-like protein containing a CARD (ASC) and recruited caspase-1. There are a huge variety of inflammasomes which recognise distinct activating signals (Fig. 7). For example, NLRC4 is activated by cytosolic flagellin. AIM2 inflammasome recognises cytosolic DNA of pathogens. The most investigated inflammasome is NLRP3 inflammasome which is activated by a wide range of agents, like K^+ ions efflux caused by bacterial toxins or viroporins, cathepsin B released after lysosomal damage, viral RNA (Kelley *et al.*, 2019). Despite of their differences the outcome of inflammasome activation is a release of inflammatory cytokines and cell death.

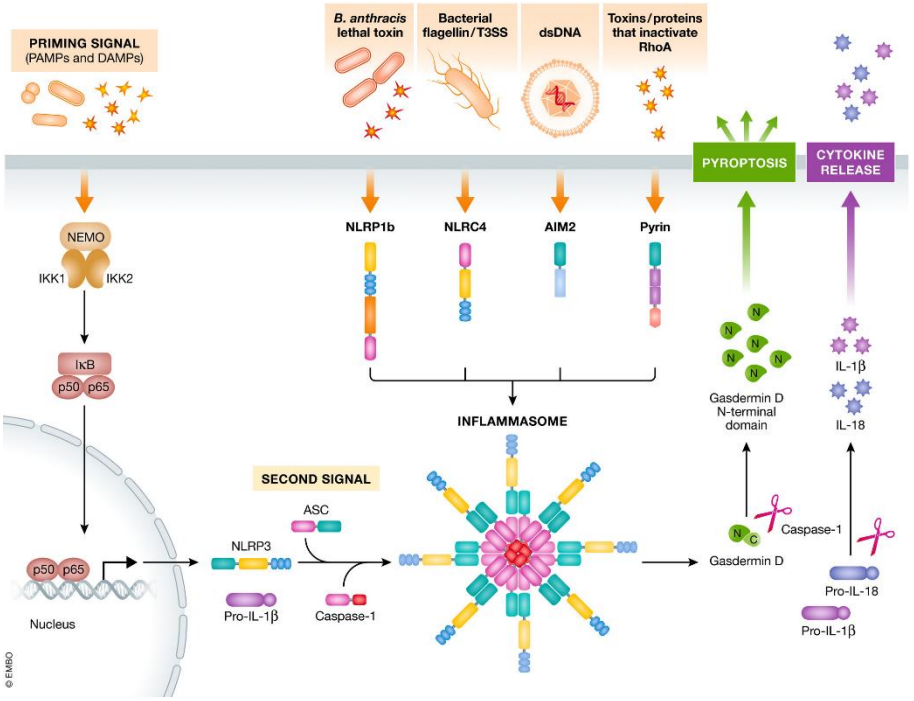

Figure 7. The structure of inflammasomes and their signalling mechanisms. Image was obtained from (Voet *et al.*, 2019).

Inflammasome can be activated by canonical or non-canonical activation pathway (Guo *et al.*, 2015). In the canonical pathway, inflammasome proteins like NOD-like protein receptors (NLR) assemble to multimeric complex

which recruit scaffold proteins like apoptosis-associated speck-like protein containing a CARD (ASC). ASC aggregate into a large protein complex, which is called "speck". ASC specks can be observed as they reach a size of around 1 μm (Dick *et al.*, 2016). Mostly, only one ASC speck forms upon inflammasome activation. ASC serves as a platform for pro-caspase-1 recruitment. Once several pro-caspase-1 are assembled, autocatalytic cleavage occurs generating active caspase-1. Then, caspase-1 cleaves its substrates, like a pro-interleukin-1 beta (pro-IL-1β), pro-IL-18 or pore forming gasdermin D (GSDMD), to their active forms. Often, but not necessarily, the outcome of inflammasome activation is led not only to the inflammatory molecule release but also to the cell death called pyroptosis – inflammatory cell death (Yu *et al.*, 2021). In the non-canonical pathway, inflammasome activating agent like intracellular/cytosolic lipopolysacharide (LPS) of gram-negative bacteria activate caspase-11 in mouse and caspases-4/-5 in humans inducing their assembly into multimeric star-shape complex (Downs *et al.*, 2020). This complex cleaves caspase-1 and GSDMD causing pyroptosis. These processes can trigger activation of canonical NLRP3 inflammasome resulting in the inflammatory cytokine secretion.

The mostly studied inflammasome is NLRP3. It needs priming before the activation to induce expression of inflammasome components (McKee and Coll, 2020). For example, LPS binding to TLR4 activate NF-κB pathway and initiate expression of NLRP3 proteins. Second signal is required for the NLRP3 inflammasome activation leading to the formation of inflammasome complex.

1.5.2. NLRP3 inflammasome and its activation pathways

NLRP3 inflammasome is the most characterised inflammasome and it can be activated by a huge variety of molecular patterns, therefore, this inflammasome activation is discussed in details. NLRP3 inflammasome activating agents can be cathepsin B released after lysosomal damage, reactive oxygen species generated after mitochondrial damage, lipid raft disturbance, pore forming toxins or other agents causing ion, such as K^+, efflux (Fig. 8) (Kelley *et al.*, 2019). The most characterised activation process is K+ efflux. This efflux is detected by P2X7 receptors and trigger cell signalling which induce inflammasome assembly. Pore forming toxins, like nigericin, are one of the most common K+ efflux inducers. Another mechanism is via generation of mitochondrial reactive oxygen species. Intracellular receptors detect these reactive oxygen species and induce inflammasome activation. Increase in intracellular Ca2+ ions also can induce NLRP3 inflammasome assembly.

Cathepsins, especially cathepsin B, released after lysosomal damage is the other activator of NLRP3 inflammasome (Campden and Zhang, 2019). Once cathepsins enter cytoplasm, they bind to cellular receptors and trigger cell signalling leading to inflammasome activation. Interestingly, one study showed that cathepsin B is required for NLRP3 activation process, including caspase-1 activation, IL-1β production and ASC speck formation, by a variety of agents, such as ATP, nigericin or crystals (Chevriaux *et al.*, 2020). NLRP3 activators mediate cathepsin B interaction with NLRP3 at the endoplasmic reticulum leading to caspase-1 activation. Despite of the fact that not all receptors of activating agents are known, the latter activators are direct or intermediate signalling molecules.

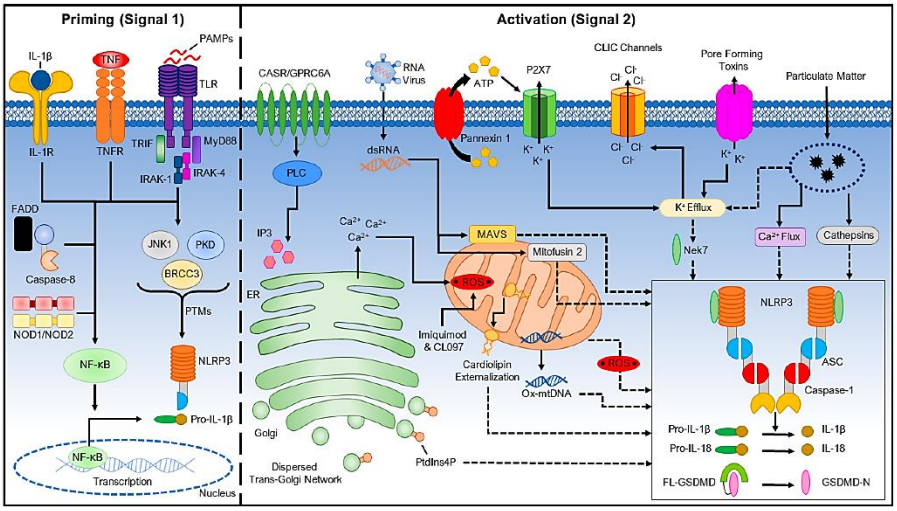

Figure 8. NLRP3 inflammasome activation pathways. In canonical inflammasome activation, signal 1 indue synthesis of inflamasome components and signal 2 triggers inflammasome complex assembly. This complex recruits caspase-1, which generates active inflammatory cytokines and GSDMD pore formation leading to pyroptosis. Abbreviations: caspase recruitment domain (CARD), chloride intracellular channel protein (CLIC), gasdermin D amino-terminal cell death domain (GSDMD-N), BRCA1/BRCA2-containing complex subunit 3 (BRCC3), protein kinase D (PKD), phospholipase C (PLC), JUN N-terminal kinase 1 (JNK1), NIMA (never in mitosis gene a)-related kinase 7 (NEK7), nuclear factor-κB (NF-κB), cation channel P2X purinoceptor 7 (P2X7), phosphatidylinositol-4-phosphate (PtdIns4P), pyrin domain (PYD), reactive oxygen species (ROS), Toll-like receptor (TLR), tumour necrosis factor (TNF), TNF receptor (TNFR), FAS-associated death domain protein (FADD), mitochondrial antiviral signalling

protein (MAVS), oxidized mitochondrial DNA (Ox-mtDNA), reactive oxygen species (ROS). Image was obtained from (Kelley *et al.*, 2019).

Particle shape and size are important not only for their phagocytosis but also for the inflammasome activation. It was shown that textured (budding) particles, made of polystyrene coated with poly-ethylene oxide, induce stronger IL-1β than smooth particles (Vaine *et al.*, 2013). For this experiment caspase-1 and NLRP3 deficient cells were used demonstrating inflammasome dependent cell activation. The higher size of the particles, the stronger IL-1β release was detected. In addition, small spherical particles (0.5-1 μm in diameter) seem to be less prominent inducers of IL-1β compared to budding particles which have 1-2 μm in diameter buds.

The properties of microbe surface are crucial for innate immune cells, like macrophages, activation. The response of the innate immunity is the first defence reaction to pathogens and pathogenic materials and may fate the further immune response. It was demonstrated that spiky TiO_2 microparticles induce mechanical stress on macrophages and dendritic cells during phagocytosis and this triggers potassium efflux followed by inflammasome activation (Wang *et al.*, 2018b). The spiky particles were internalised rapidly, however, less efficient than rough particles, thus, the spikes might disrupt phagocytosis and induce cellular stress. In addition, the spiky microparticles induced inflammasome activation by K+ efflux mechanism probably mediated by mechanical stress on the membrane. The mechanosensitive K+ channels are located on the cell membrane and after their activation induce changes in the intracellular K+ concentration mediating conformational change in NLRP3 proteins followed by inflammasome assembly (Compan *et al.*, 2012). This is an example of another mechanism how the inflammasome could be activated. In the latter research, neither cathepsin B nor reactive oxygen species were the mediators of inflammasome activation, although inflammasome triggered by phagocytosed particles was linked to the lysosomal damage.

During infection, proteases released outside the cells could produce DAMPs. For example, metalloproteinases degrade extracellular matrix and lead to its remodelling (Lu *et al.*, 2011). Extracellular matrix breakdown products can act as DAMPs and activate NLRP3 inflammasomes as it was shown investigating DAMPs associated to myocardial infarct (de Haan *et al.*, 2013). Therefore, DAMPs, generated in sterile inflammatory reactions, could be self-activating agents for NLRP3 inflammasome triggering.

Crystallopathies, induced by crystals of environmental and metabolic origin, are characterised by inflammation and cell death leading to organ

failure (Mulay and Anders, 2016). Crystalline particles, such as cholesterol crystals, activate NLRP3 inflammasome, however, inhibitors of cathepsins reduce only IL-1β release and do not rescue from cell death (Rashidi *et al.*, 2020). The mechanism of cell death induced by these particles is unclear although NLRP3 inflammasome role is evident. Using a broad range of crystalline particles of different size, shapes, and material for example, calcium phosphate, silica, titanium dioxide, cholesterol, and monosodium urate, it was demonstrated that all these particles induce caspase-independent cell death (Honarpisheh *et al.*, 2017). Receptor interacting protein kinase-1 (RIPK1), RIPK3, and mixed lineage kinase domain-like (MLKL) were identified acting in concert to induce programmed inflammatory cell death – necroptosis. In addition, phagocytosis of crystalline particles acted in line with other pathways inducing cytotoxicity as cythochalasin D prevented from cell death. Interestingly, the crystalline particle size and shape had no influence on cell activation.

NLRP3 inflammasome is a principal detector of cell stress sensing microbial and endogenous products (Kelley *et al.*, 2019). This inflammasome contributes to an ever-increasing range of inflammatory diseases. Together, this knowledge has revolutionized the field of innate immunity and has led to a deeper understanding of disease pathogenesis and, excitingly, to the development of promising therapeutics targeting the inflammasomes (Li *et al.*, 2021). However, until specific inhibitors of inflammasomes could be used in clinical practice, research on inflammasome activation mechanisms are needed to define in which disease conditions the inflammasome is activated so that its inhibition would have a beneficial effect.

1.5.3. Cathepsins

Cathepsins represent protease family having a huge variety of members classified according to their structure, protein target and catalytic mechanism (Yadati *et al.*, 2020). Mostly, they are lysosomal proteases which are activated at low pH by proteolytic cleavage. Cathepsins are important for many functions of the cell, such as peptide synthesis, digestion, adipogenesis, blood coagulation, apoptosis, and immune response. So, they are vital for the cell and play an important role in the development of immune response. After cell damage, cathepsins are released outside the cells where they degrade extracellular matrix initiating detrimental processes, including tumor metastasis or degeneration of neural tissue. So, they are important for maintaining cellular homeostasis and their dysregulation are related to

numerous diseases, like arthritis, periodontitis, atherosclerosis, obesity, AD, and schizophrenia (Patel *et al.*, 2018).

Lysosomes play a key role in regulating cell homeostasis. Lysosomes contact with other cellular organelles, such as endoplasmic reticulum, mitochondria, peroxisomes, Golgi complex, ribosomes (Ballabio and Bonifacino, 2020). This interaction has functional consequences, for example, contact with peroxisomes mediates cholesterol transfer, and contact with mitochondria regulates their fission. Lysosomes act as mediators and regulators of certain cell signalling pathways. They sense the nutrition in their surrounding and adopt according to received signal. After their response to environmental changes via surface receptors or damage, lysosomes can initiate activation of cellular processes. For example, Ca2+ released from lysosomes induce several cellular processes, including lysosomal formation from other organelles, endosome-lysosome fusion, and autophagosome-lysosome fusion.

Lysosome damage depending on its strength can lead to its repair or lysophagy. Lysosome disturbance due to their inability to degrade cargo can cause inflammation. A classic example is lysosome damage leading to its leakage and cathepsin release. Cathepsins then induce programmed cell death or other forms of cell death, like pyroptosis. Lysosomes have TLRs, TLR3, TLR7/8, and TLR9 on their surface, thus, they sense microbial nucleic acids and trigger inflammation. Protein aggregation may also affect the lysosomal-autophagic pathway, which causes the accumulation of protein aggregates and their toxicity. For example, in Huntington's disease, the huntingtin protein aggregates disrupt autophagosome biogenesis (Wong and Holzbaur, 2014), whereas in Parkinson's disease α-synuclein aggregates cause lysosomal rupture and, as a consequence, the released cathepsin B increases generation of reactive oxygen species (Freeman *et al.*, 2013). Lysosomal dysfunction caused by amyloid beta was also demonstrated in AD (Nixon, 2017). It was demonstrated that cathepsin B, expressed by microglia, contributes to neuroinflammation and is implicated in the pathogenesis of AD (Lowry and Klegeris, 2018). These examples draw attention to the importance of studies on lysosome activity searching for cell modulating targets.

Lysosomal leakage can also be a normal and controlled process in the cell. After permeabilization of lysosomal membrane, lysosomal hydrolases are delivered to specific subcellular sites of action. In this way, they control essential cellular processes, including mitosis, inflammatory signalling, and cellular motility (Stahl-Meyer *et al.*, 2021). One example is related to the inflammasome activation. Molecules released during lysosomal leakage can

activate NLRP3 inflammasome by multiple mechanisms, including cathepsin-induced K+ efflux and Ca2+-initiated signalling events (Kelley *et al.*, 2019).

1.5.4. Oligomeric proteins as potential DAMPs

Aβ is one of the most known DAMPs (Venegas and Heneka, 2017). It is generated via proteolytic cleavage of amyloid precursor protein (APP) via amyloidogenic or non-amyloidogenic pathways (Fig. 9). The generated peptide is composed of either 40 or 42 amino acids (a. a.). Especially 42 a. a. peptides are inclined to aggregate into oligomers and they are more neurotoxic than the oligomer of 40 a. a. peptides (Kuperstein *et al.*, 2010). In addition, changes in Aβ42/Aβ40 ratio predestine aggregate formation.

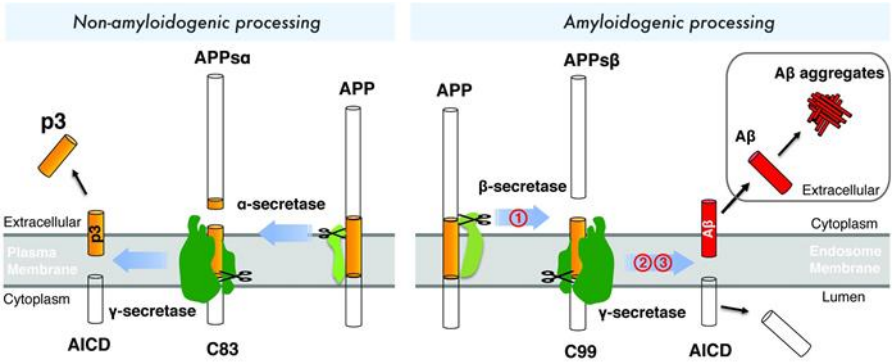

Figure 9. A schematic representation of Aβ generation. Aβ peptide is generated from precursor protein APP by proteolytic cleavage in amyloidogenic and non-amyloidogenic pathways. Image was obtained from (Zhao *et al.*, 2020).

Under physiological conditions, produced Aβ peptides are removed by microglia cells and by transfer into local blood vessels (Heneka *et al.*, 2015b). However, under pathologic conditions Aβ peptides accumulate in the brain and form aggregates (Fig. 10), which induce chronic inflammation after their encounter with immune cells (Heneka *et al.*, 2015a). Depending on physical and chemical conditions these Aβ can aggregate to fibrillary, annular or spherical oligomers (Kayed and Lasagna-Reeves, 2013).

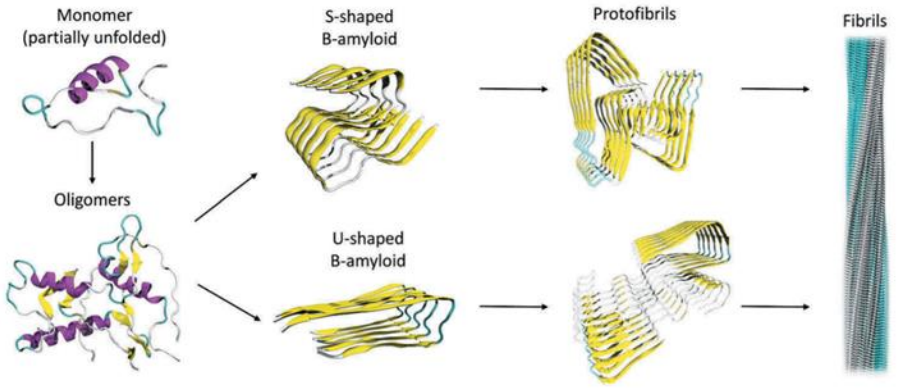

Figure 10. Representation of the structures of Aβ monomers, oligomers, protofibrils and fibrils. Aβ prefibrillar oligomers consist of dynamic beta-sheet structures. The strong hydrophobic interaction leads to tight amyloid aggregates. Image was obtained from (Grasso and Danani, 2020).

Oligomers of different size and shape activate immune cells in diverse pathways. These aggregates are recognised by the cells through various receptors, such as formyl peptide receptor-like protein 1 (FPRL1) or scavenger receptor for advanced glycation end-products (RAGE), class A1 scavenger receptors (SR-A1), macrophage receptor with collagenous structure (MARCO), CD36, alpha-2-macroglobulin receptor (Jarosz-Griffiths *et al.*, 2016). Aβ and receptor complexes are endocytosed by the cells. Most Aβ is degraded by endosome/lysosome system. However, Aβ in the lysosome can leak out to the cytosol by lysosomal membrane damage. Further, cytosolic Aβ can be degraded by the proteasomal degradation system or intracellular metalloproteinase. However, in certain cases they are hard to degrade, thus, they cause cellular stress, activate pattern recognition receptors and induce a strong inflammatory response (Mandrekar-Colucci and Landreth, 2010).

Aβ oligomers can induce the activation of both microglia (increase in CD68 expression, TNF-α and IL-6 release) (Sondag *et al.*, 2009; Dhawan *et al.*, 2012) and astrocytes (Hou *et al.*, 2011) (Fig. 11). Aβ aggregates also directly damage the cells, for example, they induce synaptic dysfunction in neurons (Mucke and Selkoe, 2012). Structural importance of Aβ aggregates on cell activation is demonstrated in studies showing that Aβ fibrils induced higher neurotoxicity than oligomers, and neurons co-culture with microglia cells increased their death after treatment with Aβ oligomers and fibrils in mouse model (Sondag *et al.*, 2009). Other study demonstrated higher neuronal cell death induced by Aβ oligomers compared to fibrils in rat neuronal-glia

culture (Cizas *et al.*, 2010). Therefore, not only size dependent neurotoxicity of Aβ aggregates exist but also different cell activation in glia cells.

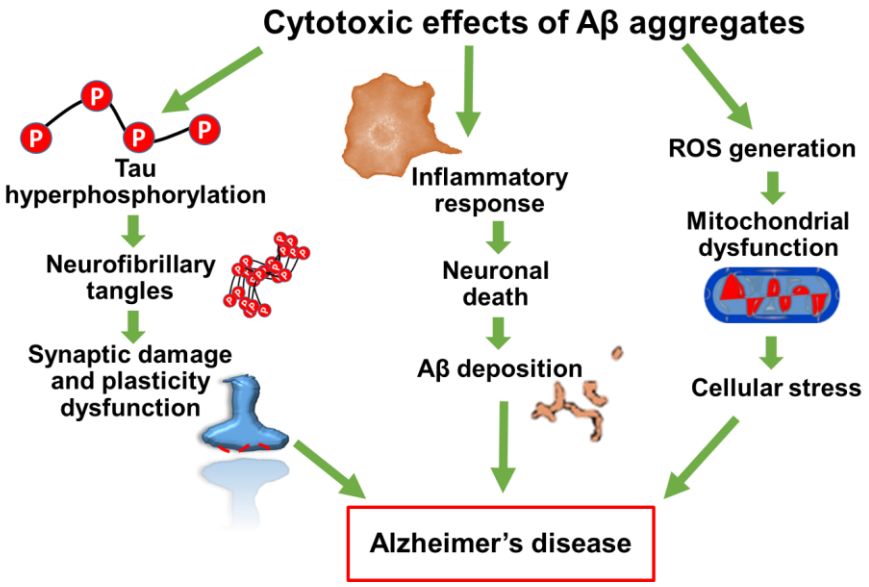

Figure 11. Mechanisms of Aβ cytotoxicity. Soluble Aβ can interact with certain receptors and activate cell signalling pathways leading to reactive oxygen species, τ protein hyperphosphorylation, inflammatory responses, which may result in neurotoxicity and the development of AD. Image was prepared usig information provided at (Chen *et al.*, 2017).

Structural properties of amyloids determine their induced cellular response, although, controversial data on microglia activation by diverse Aβ aggregates exist maybe due to different Aβ preparation conditions. In one study, fibrillar Aβ aggregates decreased cell viability while oligomers had no effect. However, oligomeric Aβ induced higher NO, TNF-α and IL-1β release (Pan *et al.*, 2011). Another study also demonstrated NO production and NF-κB activation induced by Aβ oligomers, however, Aβ fibrils did not stimulated NO production (Maezawa *et al.*, 2011). In addition, Aβ protofibrils but not monomers induced TNF-α secretion in microglia (Paranjape *et al.*, 2013). Another study revealed that Aβ oligomers, fibrils and protofibrils but not monomers induced TNF-α secretion, however, oligomers stimulated microglia more effectively than fibrils and protofibrils had the highest effect (Paranjape *et al.*, 2012). In another study, differences between Aβ oligomers and fibrils at gene expression level were demonstrated (Michelucci *et al.*, 2009). Oligomers stimulated higher expression of inflammation-related genes

encoding IL-1β, IL-6, TNF-α, COX-2, and CCL2 than fibril forming structures. Overall, Aβ induced microglia activation is evident, however, impact of structural properties of Aβ aggregates on cell activation is not fully appointed. Binding of several receptors to fibrillar Aβ was identified. The examples are SR-B receptor CD36, α6β1-integrin, and the integrin-associated protein CD47. Activation of these receptors by Aβ mediates inflammatory response (Bamberger *et al.*, 2003).

The structural properties of Aβ define its toxicity. For example, pre-fibrillar forms are assumed to be intermediates in synaptic dysfunction during AD (Chen *et al.*, 2017). Moreover, Aβ oligomers could facilitate formation of membrane pore (Sakono and Zako, 2010). These pores mediate disturbance of cellular homeostasis by inducing aberrant ion flow. In addition, Aβ oligomers activate NMDA glutamate receptors and disturb calcium homeostasis by mediated intracellular Ca2+ overload, resulting in cellular stress and synaptic loss (Texidó *et al.*, 2011).

Next to Aβ, another DAMPs, such as S100 proteins and HMBG1 exist. They are able to induce further inflammatory response by inducing upregulation of inflammatory cytokines, like TNF (Clark and Vissel, 2015). Therefore, Aβ could be one of inflammatory response initiator and AD progression depends on other DAMPs what explains why in the elderly disease severity does not always correlates with Aβ levels or why removal of Aβ by immunotherapy does not change cognitive functions and does not suspend disease progression (Nicoll *et al.*, 2019). In addition, positron emission tomography revealed that in progressed AD cortical Aβ load reaches a plateau (Ismail *et al.*, 2020). Then, tau tangle accumulation rises, however, how Aβ plaques initiate this process is unclear.

Another well characterised aggregate forming protein is tau protein (Mandelkow and Mandelkow, 2012). Normally it functions as microtubule stabilizer, however, in disease conditions certain tau isoforms can aggregate to harmful oligomers. These aggregates accumulation can directly damage the neurons causing neurodegeneration (Jouanne *et al.*, 2017). In addition, microglia as phagocytic cells internalise tau aggregates and degrade them. However, cells incapable to digest these aggregates start the spreading of pathological tau by secreting them. Tau aggregates packed into exosomes are transferred to neurons and induce their death as it seems exosomes contain the most toxic forms of tau (Brunello *et al.*, 2020).

There are even more protein aggregates forming proteins like prions (Fontaine and Brown, 2009). They are resistant to proteases and induce aggregation of other proteins thus damaging their surroundings and causing diseases. Physiologically important proteins, like insulin, can also form

aggregates, especially when it is used as a drug (Li and Leblanc, 2014). Proteins which tend to form aggregates are called amyloidogenic. There are more amyloidogenic proteins impairing cellular function and causing pathologies, for example, α-synuclein or mutated huntingtin protein (Ross and Poirier, 2004).

Proteins mentioned above can kill the neuronal cells directly or induce immune response (Kumar *et al.*, 2016). Extracellular protein aggregates in neurons cause apoptosis while intracellular damage cells by interfering their homoeostasis due to the membrane disruption mediated by ion-channels disturbance (Behl *et al.*, 1994; Gonzalez-Garcia *et al.*, 2021). They also promote oxidative stress resulting in protein and lipids oxidation, increase in intracellular calcium and mitochondrial damage (Behl *et al.*, 1994; Hu *et al.*, 2019). Focusing on microglia, protein aggregates can bind to cell surface receptor directly or after their internalisation they can be recognised by intracellular receptors (Tejera and Heneka, 2016). Then, inflammatory response is induced and other immune cells from periphery are recruited to the brain sites. Incapability to degrade ingested proteins cause lysosomal disruption or other cellular stress like mitochondrial damage and production of reactive oxygen species.

Inflammasomes are molecular sensors which are inclined to recognise endogenous danger signals triggered by protein aggregates directly or indirectly and induce a cellular response (Tejera and Heneka, 2019). Therefore, the inflammasomes, especially NLRP3 inflammasome, are investigated seeking to understand cellular damage induced by protein aggregates. NLRP3 inflammasome activation in microglia was demonstrated with α-synuclein monomers and oligomers (Scheiblich *et al.*, 2021). Impairment in α-synuclein degradation was also demonstrated, though, inhibition of NLRP3 inflammasome improved its clearance. In addition, infectious pathogens play a key role in the development of neuroinflammation (McManus and Heneka, 2017). Inflammation from periphery can worsen neurodegenerative disorder or viral infections could be a primer of disease development, such as latent viral infection is related to worsen prognosis of the neurodegenerative disease. Peripheral blood mononuclear cells from cytomegalovirus positive AD patients secreted higher levels of IFN-γ after anti-CD3/CD28 stimulation than cells of non-infected patients, suggesting that cytomegalovirus could be an inflammatory promoter in AD (Westman *et al.*, 2014). Likewise, other viruses could exacerbate neuroinflammation.

1.5.5. Inflammasome activation by protein oligomers

The inflammasome was shown to be activated by various aggregates of proteins, such as fibrillar α-synuclein (Codolo *et al.*, 2013), tau monomers and oligomers (Ising *et al.*, 2019), prion fibrils (Jen *et al.*, 2020), aggregates of synthetic islet amyloid polypeptide, which is normally co-secreted with insulin from the pancreatic β-cells (Westwell-Roper *et al.*, 2013), amyloid-beta (Aβ) fibrils (Halle *et al.*, 2008a). So, Aβ is one of the DAMPs examples which can participate in the sterile inflammatory response. Aggregates of Aβ peptides are a hallmark of AD (Murphy and LeVine, 2010). Aβ can damage neurons and activate other cells, like brain immune cells microglia, and induce inflammatory reaction (Sarlus and Heneka, 2017). Depending on its structural and physical properties Aβ oligomers of various structure, like small oligomers, protofibrils or fibrils, activate macrophages in different ways. Aβ fibrils have been implicated as inflammasome activators (Venegas *et al.*, 2017). Aβ together with ASC specks, which are formed after inflammasome activation, accelerate inflammatory response (Friker *et al.*, 2020). In addition, Aβ and ASC specks aggregates have been found in AD patients and animal models showing Aβ as one of the neuroinflammation drivers (Venegas and Heneka, 2019).

NLRP3 inflammasome implication in the neurodegenerative disease was already demonstrated (Heneka, 2017). In APP/PS1 mouse model of AD knockdown of NLRP3 gene significantly protected from memory impairment, Aβ deposition and inflammatory response measured by IL-1β level (Heneka *et al.*, 2013). In another study NLRP3 inhibitor MCC950 significantly reduced memory loss and microglia activation in an Aβ oligomer infusion model of rat hippocampus, thus, restoring features of early stage of the disease (Fekete *et al.*, 2019). In APP/PS1 mice MCC950 also reduced microglia activation and stimulated Aβ phagocytosis reducing Aβ plaque load in the hippocampus and resulting in improved cognitive functions (Dempsey *et al.*, 2017). In AD amyloidosis model of rat overexpressing Aβ, MCC950 restored synaptic plasticity in hippocampus (Qi *et al.*, 2018). As NLRP3 inflammasome is associated with numerous inflammatory diseases, neurological, cardiovascular, immune system disorders and infections, many clinical trials are ongoing using different inflammasome inhibitors (Corcoran *et al.*, 2021).

1.5.6. Inflammasome activation and inhibition by viral proteins

Inflammasome activation by certain viral proteins has been demonstrated in several studies. For example, SARS-CoV-2 (Pan *et al.*, 2021) and Zika virus

N proteins (Wang *et al.*, 2018c) activated NLRP3 inflammasome. Viroporin 2B of foot-and-mouth disease virus (*Picornaviridae* family) also was demonstrated to induce NLRP3 inflammasome activation through the increase of intracellular Ca2+ ion. Viroporins of most RNA viruses are known as inflammasome inducers, for instance, influenza virus M2 viroporin and the human rhinovirus 2B viroporin (Choudhury *et al.*, 2021). However, the mechanisms how viral proteins induce inflammasome assembly or modulate inflammatory response remain largely unknown. Proteins of certain viruses can also act as inflammasome inhibitors to prevent immune response (Fig. 12). For instance, virulence factor V protein of measles virus is known to block NLRP3 assembly (Gregory *et al.*, 2011). Another example is NS1 protein of influenza virus. Its mutation restored IL-1β release and caspase-1 activation showing NS1 inhibitory effect (Stasakova *et al.*, 2005). NS1 also impairs pro-inflammatory cytokine gene expression and was demonstrated to interact directly with NLRP3 domain (Chung *et al.*, 2015). The structural properties of proteins determining cell activation response are barely investigated and need broader understanding to predict possible outcomes of infection.

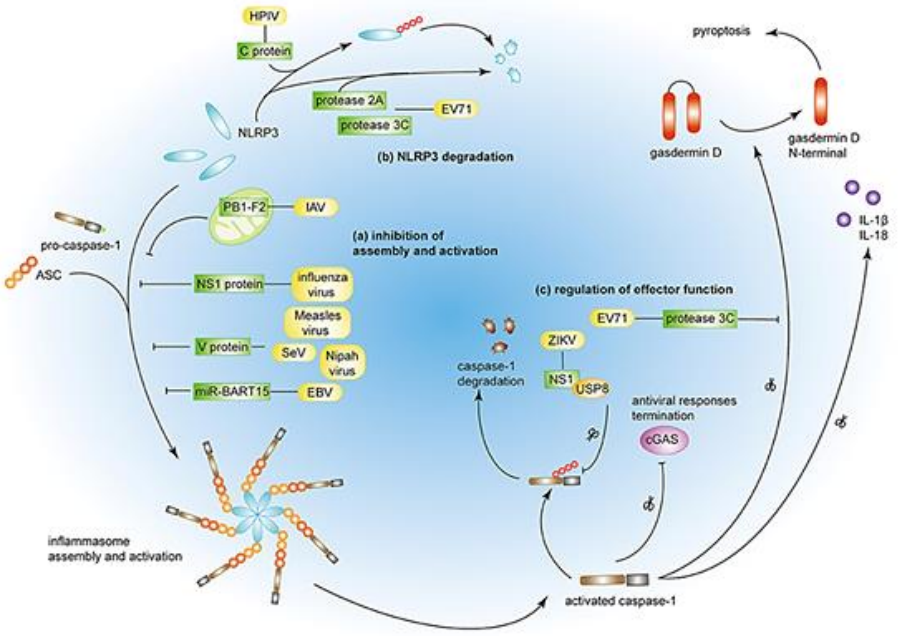

Figure 12. NLRP3 inflammasome targets used by certain viruses to evade immune response. Examples of paramyxovirus – Sendai virus, Nipah virus and measles virus. Abbreviations: hepatitis C virus (HCV), human immunodeficiency virus-1 (HIV-1), influenza A virus (IAV), Zika virus

(ZIKV), respiratory syncytial virus (RSV), human parainfluenza virus (HPIV), Sendai virus (SeV), Epstein-Barr virus (EBV), enterovirus 71 (EV71), non-structural protein 1 (NS1); host deubiquitinase USP8, DNA sensors cyclic GMP-AMP synthase (cGAS). Image was obtained from (Zhao and Zhao, 2020).

1.6. Features of polyomaviruses and paramyxoviruses

1.6.1. Polyomaviruses

Polyomaviruses (PyV, family *Polyomaviridae*) are double-stranded DNA non-enveloped viruses (Moens *et al.*, 2017; Ehlers *et al.*, 2019). They have icosahedral capsid composed of structural proteins VP1, VP2 and VP3 (Fig. 13). VP1 is important for virus interaction with cell surface receptors while VP2 and VP3 have a role in virus entry to the nucleus (Bennett *et al.*, 2015). The major protein VP1 forms pentamers which assemble into spherical particles of icosahedral structure (Fig. 14). Due to this property it is possible to produce recombinant VLPs of major capsid protein VP1 representing PyV capsids (Li *et al.*, 2015).

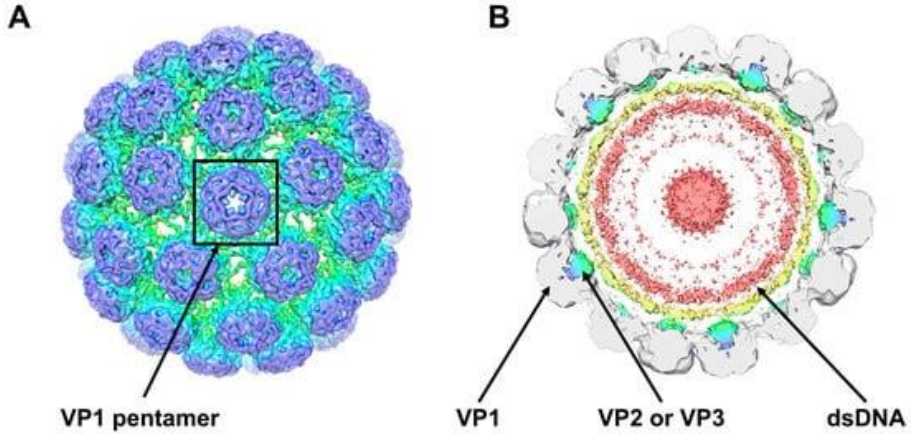

Figure 13. The structure of polyomavirus virion. Cryo-electron microscopy structure of BK PyV virion: (A) external view and (B) internal view. Image was obtained from (Helle *et al.*, 2017).

VLPs can be used to study PyV immunogenicity and virus biology. VLPs of PyV are recognised by antigen presenting cells and can induce their activation, likely, in the same way as native virus binding to cellular receptors (Gedvilaite *et al.*, 2006; Simon-Santamaria *et al.*, 2014). PyV enter the cells by binding to cell surface glycans via VP1 followed by endocytosis and disruption of endosomes (Stehle and Harrison, 1997; Smith and Helenius,

2004; Marsh and Helenius, 2006). When the virus enters the endoplasmic reticulum, structural changes of viral capsid occurs and PyVs are transported through the cytosol to the nucleus for replication. Different PyVs enter the cell via a variety of endocytosis pathways: by caveolin dependent or independent endocytosis (simian virus 40, BKPyV, murine PyV, and MCPyV), clathrin-mediated endocytosis (JCPyV), and micropinocytosis when the virus enters as extracellular vesicles independently from cell surface receptors (BKPyV and JCPyV). Caveolin-dependent endocytosis is mediated by the activation of PyV specific integrins or gangliosides in lipid raft-rich regions, resulting in the recruitment of caveolin and receptor tyrosine kinase activity. Clathrin-mediated endocytosis involves PyV activation of cellular receptors, located in lipid raft-rich regions, clathrin, dynamin and β-arrestin recruitment (Mayberry *et al.*, 2021). Receptor-mediated but caveolin- and clathrin- independent PyV endocytosis pathway is the least investigated and is lipid raft-mediated endocytosis (Mayberry and Maginnis, 2020). This pathway involves tyrosine-kinase activity and cholesterol. It is independent of dynamin. VP1 protein plays an important role in virus entry and as viral surface protein is recognised by immune cells. Therefore, VLPs of VP1 could be used to study these processes.

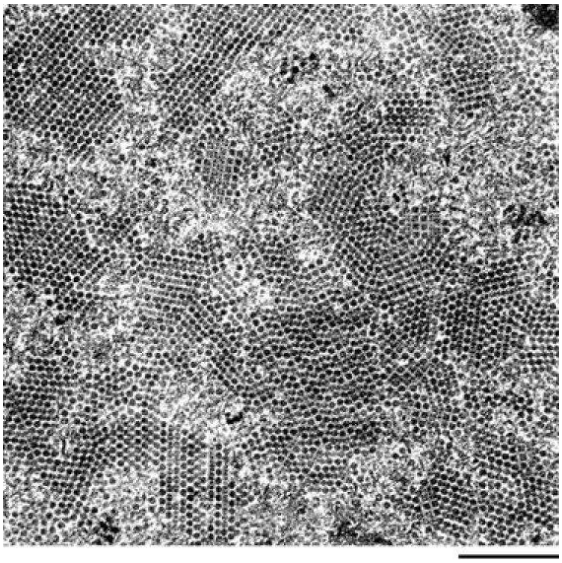

Figure 14. Electron micrograph of JC PyV virions. The image was obtained from (Shishido-Hara, 2010).

PyVs can infect both humans and animals (Ströh *et al.*, 2020). Through the time PyVs evolved by changing their VP1 to generate various virus-specific

receptor-binding sites that can specifically interact with sialylated glycans. The changes in binding sites could enable host-switching during the evolution of PyVs. Unequal hemmaglutination properties of different VLPs (also, native viruses) evince glycoprotein variation on PyV capsid surface (Major and Neel, 1998; Neu *et al.*, 2011; Neu *et al.*, 2012; Norkiene *et al.*, 2015b). Different viruses infect different animals, thus, the infection outcome could be different. Besides, certain polyomaviruses are potentially zoonotic, for example, simian virus 40 (Simon, 2008). So far, evident transmission of PyVs between different species was demonstrated for goose haemorrhagic polyomavirus (Corrand *et al.*, 2011), avian polyomavirus (budgerigar fledgling disease virus) (Johne and Müller, 1998) and bat polyomavirus species (Carr *et al.*, 2017). These evidences evince the ability of host switch among certain PyVs.

Human PyVs can enter our body via respiratory tract and get into our brain, skin or kidney (Bhattacharjee and Chattaraj, 2017). After infection, PyVs incorporate their genes into host genome and stay in the latent phase (Ling *et al.*, 2003; Doerries, 2006). According to World Health Organisation (WHO) about 50-80% of human population is infected by various PyVs during childhood (De Gascun and Carr, 2013). Most of human PyVs cause mild symptoms. Antibodies against all known 14 human PyVs were detected in human population (Kamminga *et al.*, 2018) demonstrating that these viruses are recognised by human immune system. However, immunocompromised patients are especially vulnerable to these viruses. Immune system disorders and immunosuppressive treatment could provoke virus replication and cause acute illness (Kwak *et al.*, 2002; Zanella *et al.*, 2021). Certain PyVs can modify patient cell genome and induce cancer development (Prado *et al.*, 2018). This was confirmed by detection of PyV genome in diverse types of cancer, such as skin cancer (Costa *et al.*, 2021), lung adenocarcinoma (Cai *et al.*, 2021), osteosarcoma and glioblastoma (De Mattei *et al.*, 1995). In addition, human PyVs could cause other diseases, for example, trichodysplasia spinulosa and dyskeratotic dermatitis (Ahsan and Shah, 2006). A variety of disorders emphasise the need of studying of PyV interaction with the immune system.

1.6.2. The use of VLPs as a model of viruses

VLPs represent native viruses from structural point of view, thus, they could be used for both the study of virus biology and as a platform for vaccines (Fig. 15) (Nooraei *et al.*, 2021). The examples of already established VLP-based vaccines are these: recombinant hepatitis B virus VLPs, composed of small, middle and large envelope proteins, and human papillomavirus VLPs,

composed of major capsid protein L1 (Roldão *et al.*, 2010). Vaccine composed of VP1 VLPs of goose haemorrhagic PyV was successfully generated to protect the goose from this virus infection (Mató *et al.*, 2009). In addition, VLPs can serve as vaccine platform by incorporating various antigens to VLP structure. VLPs also could be used as carriers to deliver medication to the target cells. Due to immunogenic properties of PyV VLPs they represent an attractive tool in vaccine development and biotechnology (Teunissen *et al.*, 2013). Furthermore, VLPs could be used as antigens for ELISA platforms to determine polyomavirus specific antibodies, thus, they are excellent tools for investigation of PyV seroepidemiology (Rekvig *et al.*, 2006). For instance recombinant New Jersey polyomavirus VLPs (Zhou *et al.*, 2019) and MCPyV VLPs were applied to study seroprevalence of MCPyV infection in Japanese population (Li *et al.*, 2015). In addition, generated MCPyV VLPs did not share antigenic determinants with other PyVs, BK and JC, evincing their specific use.

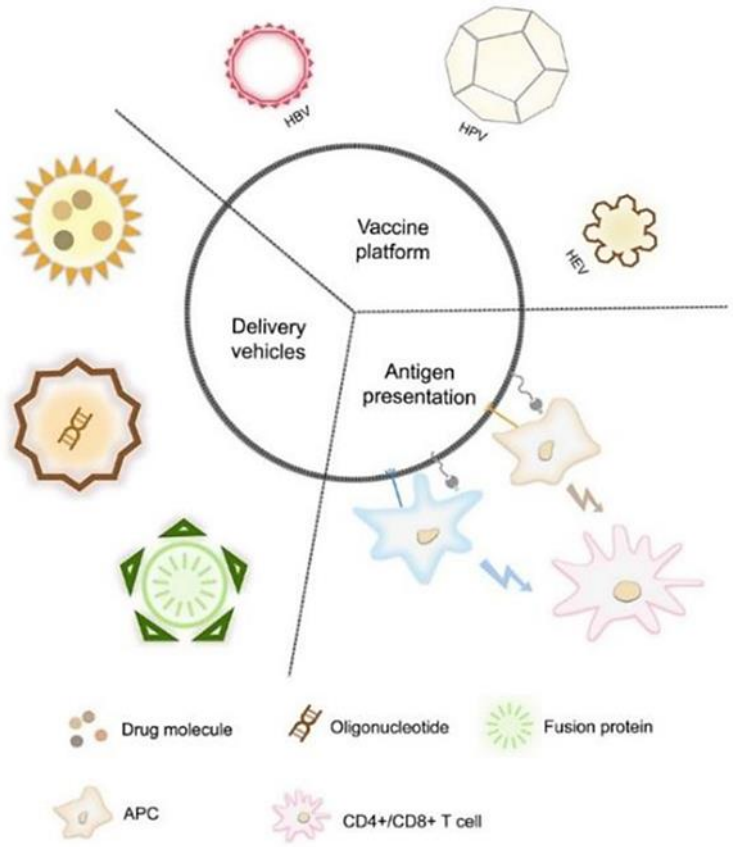

Figure 15. The application of VLPs: carriers for antigen presentation, cargo delivery, and vaccine platform. Abbreviations: antigen presenting

cells (APC), hepatitis B virus (HBV), human papillomavirus (HPV), hepatitis E virus (HEV). Image was obtained from (Qian *et al.*, 2020).

Different PyV VLPs are very similar in their spherical form and size, about 50 nm in diameter (Fig. 16). However, their amino acid sequences are different. Therefore, these VLPs (also, native viruses) bind to different receptors. The similarity is that they all bind to sialic acid containing receptors (Ströh *et al.*, 2020). Different PyV VLPs could have diverse interaction with the target cells and induce specific immune system response. For example, murine or hamster PyV VLPs activate human dendritic cells while VLPs of human BK and JC polyomaviruses and simian virus 40 induce only modest dendritic cell maturation (Gedvilaite *et al.*, 2006). Another example, VLPs of JCPyV, expressed using recombinant baculoviruses, showed high immunogenicity in rabbits while administrated with an adjuvant. However, VLPs alone did not induce immune response (Goldmann *et al.*, 1999) contrary to murine PyV (Teunissen *et al.*, 2013). Therefore, different VLPs could be used for certain purposes.

VLPs may soon be used to develop vaccine against JCPyV to reduce the risk of multifocal leukoencephalopathy (Atkinson and Atwood, 2020). This disease progresses in affected people whose immune system is insufficient to develop rapid humoral immunity against JCPyV (Ray *et al.*, 2015; Haley and Atwood, 2017). Specific VLPs could be used as a vaccine before infection and once the disease starts the developed memory B cells could rapidly produce antibodies. Murine PyV VLPs conjugated to HER-2, a proto-oncogene overexpressed in many epithelial carcinomas were successfully applied to treat cancer in mouse model (Tegerstedt *et al.*, 2005). Murine PyV VLPs are recognised by many cells from human to animal origin. Interestingly, only T cell immunity was developed in the absence of HER-2 specific antibodies. In this model, VLPs probably acted as an adjuvant non-specifically stimulating the immune system. VLPs of neurotropic JCPyV was also applied for gene therapy to treat glioblastoma where VLPs served as a delivery platform (Chao *et al.*, 2018). In another example, JCPyV VLPs were used to generate tissue-specific carrier particles by inserting a specific peptide for bladder cancer into VLPs (Lai *et al.*, 2021). The conjugate was successfully used to deliver the suicide gene to kill the target, cancer cells, by inducing apoptosis. Murine PyV VLPs were also used to deliver medication such as methotrexate to cancer cells (Abbing *et al.*, 2004). In this case VP2 fragment, binding to the hydrophobic pocket of the VP1 pentamer on the inward-facing side, was generated. Methotrexate conjugated to VP2 was bound to inward-oriented cavity of VP1 allowing encapsulation of methotrexate. The construct was

efficiently internalised and methotrexate was intracellularly released in leukaemia T cells. These examples evince VLPs as promising tools in medicine biotechnology.

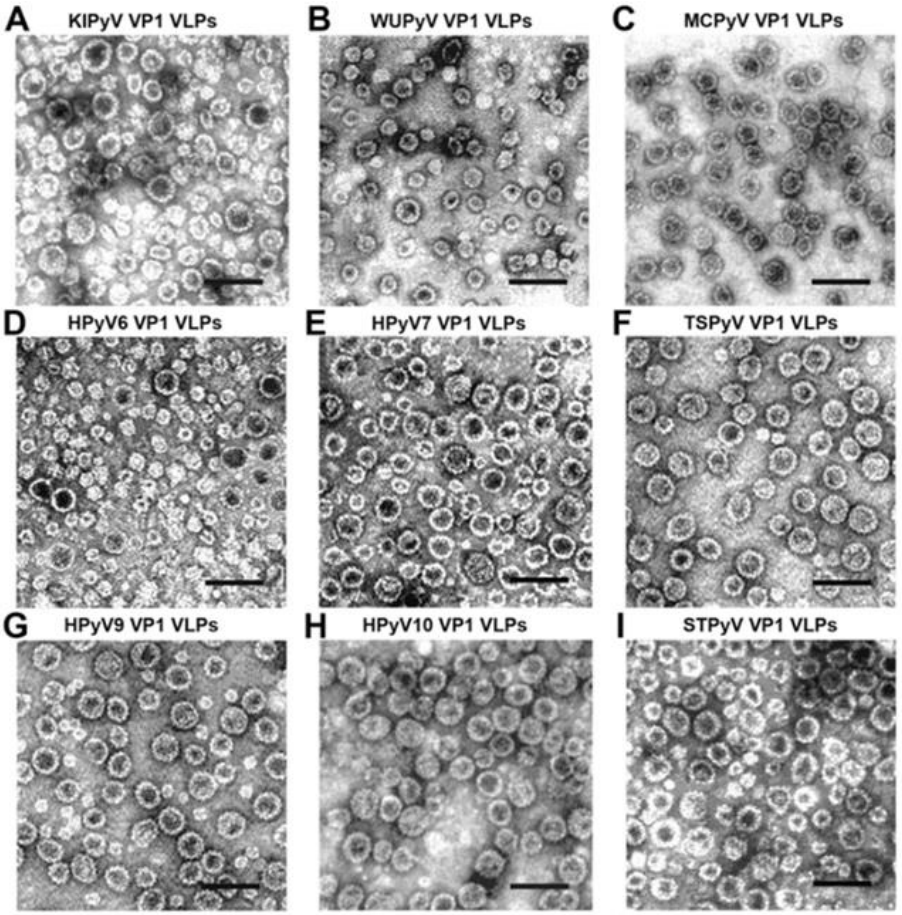

Figure 16. The structure of different human polyomavirus VLPs. Abbreviations: Karolinska Institutet polyomavirus (KIPyV), Washington University polyomavirus (WUPyV), Merkel cell polyomavirus (MCPyV), human polyomavirus 6 (HPyV6), human polyomavirus 7 (HPyV7), trichodysplasia spinulosa-associated polyomavirus (TSPyV), human polyomavirus 9 (HPyV9), human polyomavirus 10 (HPyV10), and Saint Louis polyomavirus (STLPyV). Image was obtained from (Norkiene *et al.*, 2015b). The scale bars indicate 100 nm.

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1.6.3. Paramyxoviruses

Paramyxoviruses (family *Paramyxoviridae*) are enveloped viruses about 50 to 500 nm in diameter (Cox and Plemper, 2017). The representatives of this family are measles and mumps viruses (MeV and MuV). Paramyxoviruses have single-stranded RNA genome covered by nucleocapsid (N) protein, which forms a rod-shape/tube-like structure (Fig. 17).

Paramyxoviruses enter epithelial cells via respiratory tract by binding to sialic acid receptors (Navaratnarajah *et al.*, 2020). Most of these viruses cause mild illness, however, others can cause severe diseases. Paramyxoviruses as other viruses have several mechanisms how to avoid the immune response, for example, inhibition of interferon response (Audsley and Moseley, 2013). After infection certain proteins, acting as interferon response antagonists, are encoded and they target different transcription factors. For example, V protein of MuV binds to several factors resulting to suppression of interferon expression (Xu *et al.*, 2012). V protein was demonstrated to associate with receptor-activated C kinase (RACK1) leading to interference of STAT1

interaction with interferon receptor. During viremia, structural viral protein can also spread outside the cells and may have a certain function. It was demonstrated that N protein of MeV diminishes translation of host proteins (Sato *et al.*, 2007). Measles N protein was also shown to supress immune response by binding to Fc receptors (Marie *et al.*, 2001). N protein is delivered outside the cells and inhibits the secretion of inflammatory cytokines in the surrounding cells (Marie *et al.*, 2004). Measles infection represents a risk to vulnerable individuals, children and people with immune disorders due to its ability to induce host immunosuppression (Laksono *et al.*, 2016).

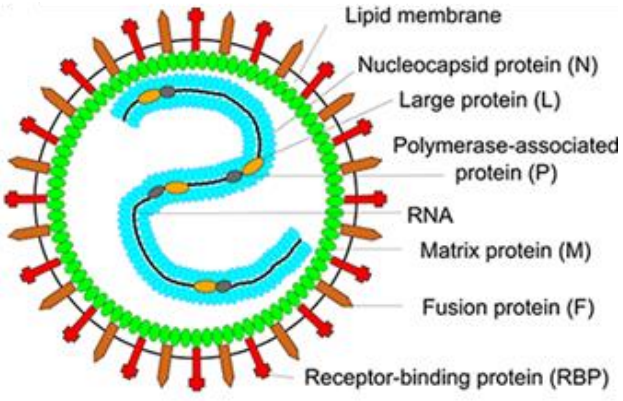

Figure 17. The structure of paramyxovirus virion. Image was obtained from (Rima *et al.*, 2019).

MuV causes inflammatory infection, such as parotitis, and it is usually a mild disease, although some patients have meningitis (Rubin *et al.*, 2015). The mumps outbreaks among MMR vaccinated people have demonstrated the limited understanding about MuV pathogenesis (Gouma *et al.*, 2016). MuV could escape both adaptive and innate immune response by the degradation of transcription factors STAT1 and STAT3 (Ulane *et al.*, 2003; Kubota *et al.*, 2005) whereas inhibition of interferon response promotes virus replication. Therefore, viral proteins serve structural and host response modifying function, however, mostly too little is known about the mechanism of action. Recombinant viral proteins could be used to study virus biology. For example, nucleocapsid-like particles (NLPs), which form "herring-bone" morphology, are similar to the native viral nucleocapsids (Fig. 18). Therefore, NLPs are excellent tools to study the immune response and N protein interaction with host immune cells, having in mind that during viremia N protein is detected outside the infected cells.

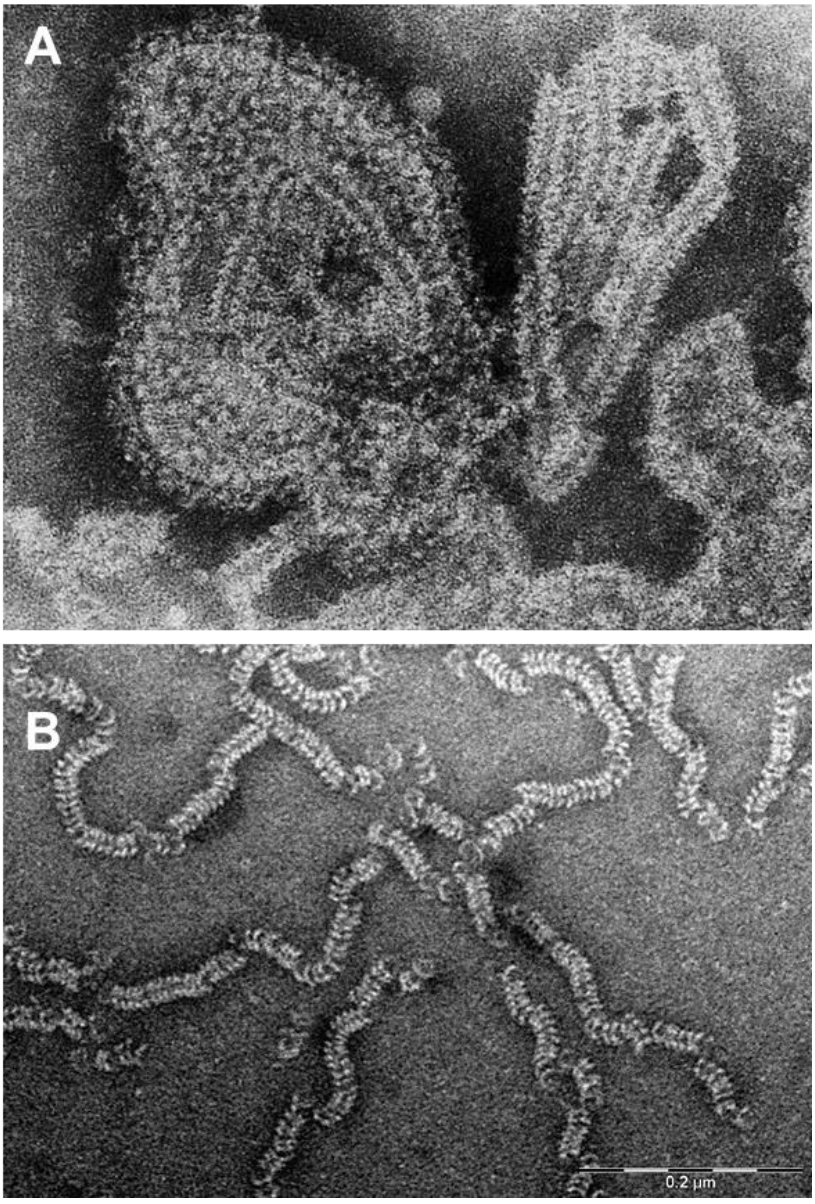

Figure 18. Electron micrographs of Paramyxovirus nucleocapsid. (A) Native MuV nucleocapsids. The virion ruptured allowing some of the nucleocapsid to escape on the right. Image was made by Frederick A. Murphy (https://phil.cdc.gov/details.aspx?pid=1874). (B) Recombinant NLPs of MeV expressed in yeast (Slibinskas *et al.*, 2004). Some of filaments degrade into annual structures. The scale bars indicate 200 nm. NLP image was made by dr. Rimantas Slibinskas and was used with his permission.

2. MATERIALS AND METHODS

2.1 MATERIALS

$A\beta_{1-42}$ peptide (ID. No.:CM1804161) was from PSL Peptide Specialty Laboratories GmbH. Dulbecco's modified Eagle's medium (DMEM; cat#61965059), Roswell Park Memorial Institute 1640 medium (RPMI, cat#61870044), FluoroBrite DMEM (cat#A1896701), fetal bovine serum (FBS; cat#26140079), penicillin/streptomycin (P/S; cat#15140122), Dulbecco's Phosphate Buffered Saline (PBS; cat#14040166) and 0.25 % trypsin-EDTA solution (cat#25200056) were obtained from Gibco, Thermo Fisher Scientific (Waltham, CA, USA). Cell culture plates: T75 culture flasks (cat#658170) were from Greiner Bio-One (Kremsmünster, Austria); T75 culture flasks Cell Culture Treated EasYFlasks (cat#156499) were from Nunc, Thermo Fisher Scientific; TPP Multi-well tissue culture plates (cat# 92012, cat#92024, cat#92048) were from TPP Techno Plastic Products AG; IbiTreat µ-slides with 8 well (cat#80826) and 96 well µ-plates (cat#89626) were from Ibidi (Martinsried, Germany). Lipopolysaccharide (LPS; cat#tlrl-eblps), nigericin (cat#tlr-nig), MCC950 (cat#inh-mcc), normocin (cat#ant-nr-1) and zeocin (cat#ant-zn-05) were from InvivoGen (CA, USA). K777 [K11777] (cat#AG-CR1-0158-M001) was from Adipogen (USA). CA-074 Me (cat#A8239) was from ApexBio Technology (USA). Materials for atomic force microscopy: TESP-V2 cantilevers were from Bruker (California, USA); mica sheets were from SPI Supplies (USA). LDH cytotoxicity detection kit (cat#11644793001) was from Roche Diagnostics, Sigma-Aldrich by Merck. Phorbol 12-myristate 13-acetate (PMA, cat# P1585-1MG) was obtained from Sigma-Aldrich by Merck. XTT Cell Viability Kit (cat#9095) from Cell Signaling Technology® (USA). Propidium Iodide (PI; cat#638), Hoechst 33342 (cat#639) and FAM-FLICA® Caspase-1 Assay Kit (containing FLICA reagent FAM-YVAD-FMK – caspase-1 inhibitor probe; cat#98) were obtained from ImmunoChemistry Technologies (Minnesota, USA). Poly-L-lysine (PLL; cat#1524), paraformaldehyde (PFA; cat#158127), 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP; cat#105228) and Triton X-100 solution (cat#X100) were obtained from Sigma-Aldrich by Merck (USA). Dimethylsulfoxide (DMSO; cat#A3672) was from PanReac AppliChem and the ITW Reagents (Spain, Italy, Germany). Membrane dye was from (cat#C10046, Invitrogen, USA). Amyloid beta peptide (Aβ; ID. No.:CM1804161) was from PSL Peptide Specialty Laboratories GmbH (Germany). IL-1β/IL-1F2 DuoSet ELISA kit (cat#DY401) and TNF-α DuoSet ELISA kit (cat#DY410) were from R&D Systems (USA). Human IL-1 beta

Uncoated ELISA Kit (cat# 88-7261-77) and TNF alpha Uncoated ELISA Kit (cat#88-7346-86), IL-6 Uncoated ELISA Kit (cat#88-7066), and IL-10 Uncoated ELISA Kit (cat#88-7106) were from Invitrogen, Thermo Fisher Scientific. Normal goat serum (cat#S-1000) was from Vector Laboratories (Burlingame, CA, USA). The primary antibodies: anti-ASC (cat#AG-25B-0006), anti-human caspase-1 (clone Bally-1, cat#AG-20B-0048) from AdipoGen (USA); rat anti-CD68 (cat#MCA1957) from Serotec by Bio-Rad, Hercules (CA, USA); mouse anti-Aβ (clone 11E12) antibody was in-house generated (Dalgediene *et al.*, 2013). Secondary antibodies: F(ab')2-Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (cat#A11017; RRID:AB_143160), Goat anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 (cat#A11007; RRID:AB_141374), F(ab')2-Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (cat#A11070; RRID:AB_142134) and Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 (cat#A11005; RRID:AB_141372) were obtained from Invitrogen, Thermo Fisher Scientific (USA). Flow cytometry reagents: cell surface receptors FcγRIII (CD16) and FcγRII (CD32) blocking solution Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block) (cat#553141; RRID:AB_394656) was from (Becton, Dickinson and Company, USA), rat APC anti-mouse/human CD11b Antibody (clone M1/70, cat#101212; RRID: AB_312795) was from BioLegend (USA); F(ab')2-Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (cat#A11017; RRID:AB_143160) was from Life Technologies, Thermo Fisher Scientific (USA). Centrifugal filters with 3 kDa a cutoff – Amicon Ultra-0.5 Centrifugal Filter Unit (cat#UFC500396) and 10 kDa a cutoff (cat#UFC501096) were from Merk Millipore (Germany). Reagents for immunoblotting using Jess System (ProteinSimple, Bio-Techne, USA): primary anti-caspase-1 antibody – anti-Caspase-1 (p20) (mouse), mAb (Casper-1) (cat#AG-20B-0042; RRID:AB_2490248) from AdipoGen (USA); 12- 230 kDa Jess/Wes Separation Module (cat#SM-W004) and secondary antibody – ready to use HRP conjugated goat anti-mouse antibody (cat#042-205) were from ProteinSimple, Bio-Techne. Tween-20 (cat# 9127.1) and sulphuric acid (H_2SO_4, cat#X873.1) were from CarlRoth. Chemiluminescent substrate – SuperSignal West Femto Maximum Sensitivity Substrate (cat#34094) was from Thermo Fisher Scientific.

2.2 METHODS

2.2.1. Primary microglia cell culture

All animals used for cell isolation were treated according to the legal and ethical requirements of the University of Bonn, Medical Center (Germany) and the North Rhine-Westphalia federal ministry for nature, environment and consumer protection (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen). Experiments in Lithuania were caried out according to European and Lithuanian legislation following the 3Rs Rule. Mice were housed for primary microglia cell preparation only which did not require further approval. Primary microglia were isolated as described before with some adaptations (Giulian and Baker, 1986). Briefly, brains from neonatal (p0–p3, mixed gender cultures) C57BL/6J (WT) mice were stripped of the meninges and dissociated using mechanical shearing and trypsin. Cells of two brains were plated on PLL coated T75 culture flasks (Greiner Bio-One) and cultivated in DMEM supplemented with 10% heat-inactivated FBS and 1% P/S (100 U/ml/100 µg/ml). On the next day, cells were washed three times with PBS to remove cellular debris and cultured with DMEM supplemented with 10 % FBS, 1% P/S and 10 % L929 conditioned medium as a source of growth factors. After approximately 8 days, loosely attached mature microglia were shaken off the astrocytic monolayer with a repetition of the harvesting procedure at every 2 to 3 days for up to 3 times. Cells were seeded at a density of 1 million/cm^2 in 1/2 old medium (condition medium from microglia shake) and 1/2 fresh DMEM supplemented with 10 % FBS and 1 % P/S and allowed to adhere overnight. On the next day microglia were primed with 50 ng/mL LPS for 3 h and treated with 2.5 µM Aβ aggregates for 6 h in a serum free DMEM containing 1% P/S. As a positive control, the inflammasome inducer nigericin was used at 10 µM concentration. MCC950, which selectively inhibits the NLRP3 inflammasome, was used at 1 µM concentration.

Cell line mouse fibroblasts L929 was cultivated in DMEM medium supplemented with 1 % P/S and 10 % FBS. L929 condition medium was used as supplement for primary microglia. Cells were seeded at 1/8 confluent in 75 cm^2 flasks and cultivated for 14 days. Then, cell culture supernatant was collected, centrifuged at 300 × g for 10 min and filtered using 0.2 µm pore size filter. Prepared condition medium was stored at -20 °C.

2.2.2. $A\beta_{1-42}$ preparation

$A\beta_{1-42}$ oligomers and protofibrils were prepared according to previously described protocol (Stine *et al.*, 2003). Briefly, Aβ was dissolved in ice cold HFIP to a final concentration of 221 μM. 100 μl aliquots were incubated at room temperature for 1 h and centrifuged in a speed vacuum centrifuge at 800 × g for 10 min until the HFIP had evaporated. The tubes with the peptide film were stored at -80 °C. Aβ pre-treated with HFIP was dissolved in DMSO to a final concentration of 2.5 mM and sonicated for 10 min. Aβ or the DMSO vehicle control was added to phenol red free DMEM to a final concentration of 100 μM and incubated for 24 h at 4 °C or 37 °C to produce oligomers (*Protocol I*) and protofibrils (*Protocol II*) respectively. To confirm that the Aβ oligomers and protofibrils were intact during cell culture treatment further analysis was performed. Aβ preparations were diluted with DMEM from 100 μM to 2.5 μM and incubated for 6 h at 37 °C and 5 % CO_2 to simulate cell culture conditions. Aβ oligomers and protofibrils were then characterised by atomic force microscopy. For cell treatment experiments buffers used for the preparation of Aβ served as vehicle control: all solutions and incubation conditions were the same except solutions without Aβ was added. They were referred as oligomers control (Con. O) or protofibrils control (Con. P).

2.2.3. Atomic force microscopy imaging

To assess the size and morphology of the $A\beta_{1-42}$ oligomers and protofibrils, atomic force microscopy measurements were performed using a Dimension Icon system (Bruker, USA) in the tapping mode in air. Imaging was performed at the room temperature using TESP-V2 cantilevers with a nominal spring constant 40 N/m. Freshly cleaved mica sheets (grade 4, SPI Supplies, USA) were modified with PLL for 10 min, then rinsed with deionised water, and dried under a nitrogen stream. Before measurement 20 μL of a 2.5 μM Aß solution was spotted on PLL modified mica surface for 10 min, then rinsed with deionised water, and dried under nitrogen stream. Images were acquired at scan rate 0.4 Hz in 512 × 512 pixel mode. Images were analyzed by v4.0 Beta 9.1 WSxM (Horcas *et al.*, 2007) and Nanoscope Analysis v1.40 (Bruker, Santa Barbara, CA) software. The mean height of Aβ oligomers and protofibrils ("z-height") was estimated by determining step-height histograms.

2.2.4. Cell viability/cytotoxicity assay

Cell viability/cytotoxicity was measured using 3 different assays:

I. To assess the cytotoxicity, an LDH cytotoxicity detection kit was used. 50 µL of cell supernatants was used to perform the cytotoxicity assay according to the manufacturer's protocol. Absorbance was measured at 450 nm using a microplate reader.

II. The metabolic activity of primary microglia was quantified using the XTT Cell Viability Kit. Primary microglia were seeded at 1 million cell/ml into 96 well plates and treated with 100 µl medium per well. After treatment with the various stimuli, the XTT Reagent and Electron Coupling Solution were added directly to the cell culture medium according to the manufacturer's protocol and incubated for 4 h. Absorbance was measured at 450 nm with a microplate reader.

III. To determine cell viability PI/Hoechst nuclear staining was used. Nuclei were stained with 1.25 µg/ml PI and 1 µg/ml Hoechst33342 in cell culture medium for 30 min. The cells were washed with PBS and fixed with 4 % PFA. The fluorescent signal was determined using a fluorescence microscope. Images were taken using a 20× objective. Viability was quantified according to a ratio of PI (dead cells) and Hoechst (all cells) normalised to control cells.

2.2.5. Measurement of cytokine release

NLRP3 inflammasome activation by Aβ was determined by measuring the IL-1β secretion using the mouse IL-1 beta/IL-1F2 DuoSet ELISA kit. To assess the extent of the inflammatory response, TNF-α release was determined using the mouse TNF-α DuoSet ELISA kit. Primary microglia cells were treated with 1 ml medium per well in 6 well plates. After treatment, the cell culture supernatants were harvested and frozen at -80 °C. Supernatants were thawed once and assayed according to the manufacturer's protocol. The optical density was determined at 450 nm photometrically with a microplate reader (Infinite M200; Tecan, Männedorf, Switzerland). The concentration of released cytokines was quantified using the relevant standard curves.

Human IL-1β, TNF-α, IL-6 and IL-10 were measured in the cell culture supernatant using ELISA kits. Procedures were carried out according to manufacture instructions.

2.2.6. Immunocytochemistry

Cells were stained in IbiTreat 8 well µ-slides or 96 well µ-plates. After treatment, cells were washed with PBS and fixed in 4 % PFA dissolved in PBS for 15 min and permeabilized with 0.1% Triton X-100 prepared in PBS for 10 min. Blocking solution – PBS containing 5% normal goat serum (1:200) was applied for 30 min followed by two washing steps. The primary antibodies rabbit anti-ASC (1:200; clone AL177), rat anti-CD68 (1:1000; clone FA-11) and mouse anti-Aβ (3.3 µg/ml; clone 11E12) were added to the blocking solution and incubated overnight. The following secondary antibodies were used respectively (1:600): goat anti-rabbit, goat anti-rat and goat anti-mouse. The secondary antibodies were applied for 2 h followed by two washing steps. Hoechst33342 was used for nuclear staining at 1 µg/mL for 30 min in PBS. ASC speck images were taken using a 60× oil-objective. Aβ oligomers and protofibrils uptake was measured by imaging with 40× objective. CD68 was used as a microglia and lysosomal marker.

2.2.7. FLICA assay (active caspase-1)

Active caspase-1 was detected using the FLICA assay according to the manufacturer's protocol. Briefly, cells were stained in IbiTreat µ-slides with 8 wells. FLICA reagent (FAM-YVAD-FMK – caspase-1 inhibitor probe) was added after the 6 h treatment with Aβ and incubated for 1 h. Cells were washed three times and stained with Hoechst 33342 at 1 µg/ml and membrane dye (1:1000). After washing the cells were fixed with 4 % PFA and washed again with PBS. Caspase-1 activation was analysed directly after staining. The signal was visualised by a fluorescence microscopy using a 60× oil-objective for mouse cells (Nikon) and 20× objective human cells (EVOS FL Auto). FLICA-caspase-1 intensity signal was measured and normalised to the number of cells. In every experiment the calculated value of FLICA-caspase-1 intensity was normalised to the value of untreated cells (control).

2.2.8. Microscopy

All experiments were examined using a Nikon Eclipse Ti fluorescence microscope (Nikon, Tokio, Japan) and EVOS FL Auto fluorescent microscope (ThermoFisher Scientific, USA). Acquired images were processed using NIS-elements 4 (Nikon) and ImageJ (Wayne Rusband; National Institute of Health, Bethesda, MD, USA).

2.2.9. Flow cytometry

To evaluate Aβ phagocytosis flow cytometric analysis was performed. Microglia that were incubated with various stimuli were collected by detaching with 0.25 % trypsin-EDTA solution for 10 min and the trypsin was then inhibited with DMEM supplemented with 10 % FBS and 1% P/S. After washing the cells were fixed with 4 % PFA for 10 min and permeabilised with 0.03 % Triton X-100 for 5 min. After washing the cell surface receptors FcγRIII (CD16) and FcγRII (CD32) were blocked with Fc block (anti-mouse CD16/32) solution. The cells were incubated with primary antibodies against Aβ (clone 11E12) overnight at 4 °C. After washing the cells were stained with rat anti-mouse CD11b-APC (1:100; clone M1/70) together with the secondary antibody goat anti-mouse (1:600) for 1 h at 4 °C. After washing microglia were resuspended in the staining solution (2% FBS in PBS). Flow cytometric data was acquired on a BD FACSCanto II flow cytometer (Becton, Dickinson and Company, USA) and analysed using FlowJo software (FlowJo, LLC, USA).

2.2.10. Immunoblotting

The quantity of mouse cleaved caspase-1 was determined by Western-blot (WB) using the Jess system (ProteinSimple, Bio-Techne, USA). This system uses cappilary electrophoresis. It is automated WB system when protein separation and immunodetection are made by a machine. Detected signal is converted to a numerical value and can be analysed by *Compass* program. This program also allows to convert detected signal into traditional WB view. Primary microglia were cultured in 6-well plates and treated at a density of 2 million cells/ml per 9.5 cm^2 growth area. The protein content of the supernatants was concentrated 10x using centrifugal filters with 3 kDa cutoff. The chemiluminescence assay was used following ProteinSimple instructions. 12-230 kDa Jess/Wes Separation Module was used and 3 µl of each sample was loaded for 9 s. The incubation time of the primary and the secondary antibodies was 30 min. As a primary antibody anti-caspase-1 was used at a concentration of 20 µg/ml (1:50 dilution). For the secondary antibody ready to use HRP conjugated goat anti-mouse antibody was used.

Human cleaved caspase-1 was determined in cell culture supernatant by WB. After cell treatment with viral proteins, the protein content of the supernatants was concentrated 10x using centrifugal filters with 10 kDa cutoff. The samples were boiled in a reducing sample buffer and separated in 4-12% polyacrylamide gel (#NW04122BOX, Invitrogen) by electrophoresis

in MES SDS running buffer (#B0002 Invitrogen). The proteins from the SDS-PAGE gel were blotted onto 0.2 µm nitrocellulose membrane (#LC2000, Invitrogen) by wet transfer. The membrane was blocked with 3% BSA in PBS for 1 h at RT and rinsed with TBST. The membrane was then incubated with primary antibodies against human caspase-1 (1:1000) in TBST with 1% BSA overnight at 4 °C. Thereafter, the membrane was incubated with secondary antibodies Goat Anti-Mouse IgG (H+L)-HRP Conjugate (Bio-Rad) diluted 1:5000 in TBST with 1% BSA for 1h at RT. The horseradish peroxidase (HRP) enzymatic reaction was developed using chemiluminescent substrate. The signal was visualised using ChemiDoc Imaging System (Bio-Rad Laboratories).

2.2.11. Human cell culture

Cell line human monocytes THP-1 were cultivated in RPMI 1640 medium supplemented with 1 % P/S and 10 % FBS. Before the experiment cells were differentiated to macrophages according to the review describing different conditions for THP-1 macrophage preparation (Chanput *et al.*, 2014). THP-1 cells were seeded in the medium supplemented with 100 ng/ml PMA and differentiated for 48 h to macrophage-like cells. Then, the medium was changed to a fresh serum medium and allowed to rest for another 24 h. Non-adherent cells were washed out with serum free RPMI and the adherent cells were treated in the serum free medium. These human monocyte-derived macrophages generated from THP-1 monocytes were further called THP-1 macrophages. For viral proteins experiments, THP-1 macrophages were treated with viral proteins at 20 µg/ml. After the treatment cell culture supernatant was collected, centrifuged at 600 × g for 10 min and supernatants transferred to the new tubes. The cells were used for several assays to test inflammasome activation.

Primary human macrophages were purchased from Lonza (#4W-700). Macrophages were derived from CD14+ human monocytes of one donor. Cryopreserved cells were thawed and cultured for two days in RPMI 1640 medium supplemented with 10% FBS and 1% P/S before treatment. The cells were treated as THP-1 macrophages in the serum-free RPMI.

2.2.12. Generation of recombinant PyV-derived VLPs and N proteins of measles and mumps viruses

Recombinant PyV-derived VP1 VLPs used in this study were generated in yeast, purified and analysed by electron microscopy as described previously

(Norkiene *et al.,* 2015a). VLPs were prepared at Department of Eukaryote Gene Engineering, Life Science Center, Vilnius University. Recombinant mumps (cat#12MuNP-ASc-Gly-C) and measles (cat#12MeN-BSc-Gly-C) N proteins were purchased from Baltymas (Lithuania). The size of recombinant protein monomer: MCPyV VP1 MW = 46.56 kDa; KIPyV VP1 MW = 41.59 kDa; MeV N MW = 58 kDa; MuV N MW = 66 kDa.

2.2.13. ASC speck detection using THP1-ASC-GFP cell line

THP1-ASC-GFP cells are inflammasome reporter cells for monitoring ASC-dependent inflammasome formation using fluorescence microscopy. These cells stably express a gene encoding an ASC-GFP fusion protein for which expression is driven by an NF-κB-inducible promoter. Upon the first step of inflammasome activation, NF-kB-dependent ASC-GFP expression is induced and observed throughout the cytoplasm. Following the second step of inflammasome activation, ASC-GFP polymerizes to form a macromolecular, micrometre-sized complex.

THP1-ASC-GFP cells were cultured in RPMI 1640 growth medium supplemented with 10% FBS, 100 U/ml/100 µg/ml P/S, 100 µg/ml zeocin, and 100 µg/ml normocin. For experiments THP1-ASC-GFP were differentiated to macrophages. The cells were incubated with 100 ng/ml PMA (24 w. pl. 1 ml/w) in RPMI 1640 medium supplemented with 10% FBS and 100 U/ml-100 µg/ml P/S. After 48 h the cell culture medium was removed and replaced with fresh serum medium for another 24 h. Prepared macrophages were washed once with serum free RPMI 1640 and treated in serum free RPMI 1640 supplemented with 100 U/ml/100 µg/ml P/S.

THP1-ASC-GFP macrophages were treated with 20 µg/ml of viral proteins for 24 h. After the treatment phenol red free FluoroBrite DMEM containing Hoechst33342 was added for 20 min to stain nuclei. Then all medium was removed and replaced with FluoroBrite DMEM. ASC specks were visualised by microscope taking at least 6 photos per well. The photos were analysed by ImageJ. The number of ASC specks and cells were counted per photo. ASC specks per cell were compared between treatments.

2.2.14. Immunocytochemistry for studying the uptake of VLPs by macrophages

The uptake of VLPs was assayed by fluorescent immunostaining. Cells were stained in IbidiTreat 96 well µ-plates. After the treatment cells were washed with PBS and fixed in 4% PFA dissolved in PBS for 15 min and

permeabilised with 0.1% Triton X-100 prepared in PBS for 10 min. Blocking solution – PBS containing 2% BSA was applied for 30 min followed by two washing steps. The primary antibodies rabbit polyclonal anti-CD68 (1:100; #25747-1-AP, Proteintech) and mouse anti-PyV VP1 VLPs (monoclonal antibodies of hybridoma supernatant at dilution 1:2) were added directly to the blocking solution and incubated overnight. The following secondary antibodies were used respectively: goat anti-rabbit (1:1000) and goat anti-mouse (1:1000). Secondary antibodies were applied for 2 h followed by two washing steps. Then nuclei were stained with Hoechst33342 (1 μg/mL) for 30 min in PBS. The images were taken using a 40× objective. CD68 was used as a microglia and lysosomal marker. The experiment was imaged using EVOS FL Auto fluorescence microscope (Thermo Fisher Scientific, USA). Acquired images were processed using ImageJ (Wayne Rusband; National Institute of Health, Bethesda, MD, USA).

For the immunocytochemistry, in-house generated murine monoclonal antibodies against recombinant NLPs and VLPs were used (#MAb clone – virus antigen indicated): #7C11 – MeV N (Zvirbliene *et al.*, 2007); #5E3 – MuV N (Samuel *et al.*, 2002); #5G8 – KIPyV VP1; #11A2 – MCPyV VP1. Cell condition medium of hybridomas was used.

Data were analysed and images were processed using ImageJ.

2.2.15. Statistical analysis

Data from at least 3 independent experiments or technical repeats were analysed by 9.2.0 GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Independent experiments referred to the number of independent cell culture preparations. Data presented as box-plots (showing minimum, first quartile, median, third quartile, and maximum) or bar graphs with individual data points. Normality test was carried out to test if the values come from a Gaussian distribution. Statistical comparisons of control versus treated cells were performed with analysis of variance (ANOVA) using multiple comparison Bonfferoni or Tukey tests. If the data had only 3 experiments, unpaired t-test was used. In the case the data did not follow Gaussian distribution, Kruskal-Wallis and Dunn's post hoc tests were used. Differences with p value less than 0.05 were considered to be statistically significant and indicated as *$p < 0.05$, **$p<0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3. RESULTS

We investigated the ability of oligomeric proteins of different structure and origin to induce inflammation focusing on inflammasome activation. Our study model was macrophage cell culture. Aβ oligomers were selected as a model of endogenous antigen and viral oligomeric proteins were used as a model of oligomers foreign to the host cells depicting viral antigens.

3.1 Investigation of microglia activation by Aβ oligomers

We started from soluble Aβ oligomers and protofibrils. We used soluble Aβ aggregates – primary amyloid derivative to test whether they induce inflammatory response as primary source of inflammation. Soluble Aβ oligomers of different structure and size were selected as representatives of different protein aggregation stages. Specific NLRP3 inflammasome inhibitor MCC950 was used to confirm NLRP3 inflammasome activation. As Aβ contributes to the neurodegenerative disease, primary murine brain macrophages – microglia – were selected as a relevant cell model.

Firstly, we characterised the structure of our Aβ aggregates using atomic force microscopy. We used two protocols to prepare Aβ aggregates: Protocol I and Protocol II. Aβ prepared according to Protocol I formed oligomers about 4 nm in diameter (Fig. 19A). Aβ preparation of protocol II aggregated to structures like a core of fibrils, called protofibrils (Fig. 19C). Next, we tested the dynamics of prepared Aβ aggregates. We diluted Aβ oligomers and protofibrils to a concentration used for cell treatment and kept at 37 °C and 5% CO_2 for 6 hrs as during cell treatment period. We detected dissociation of Aβ protofibrils to smaller structures (Fig. 19C). In Aβ oligomer preparation the scanned surface was fully covered with very small Aβ particles which were hard to scan. This means that our Aβ oligomers do not aggregate into fibril-like structures during incubation period.

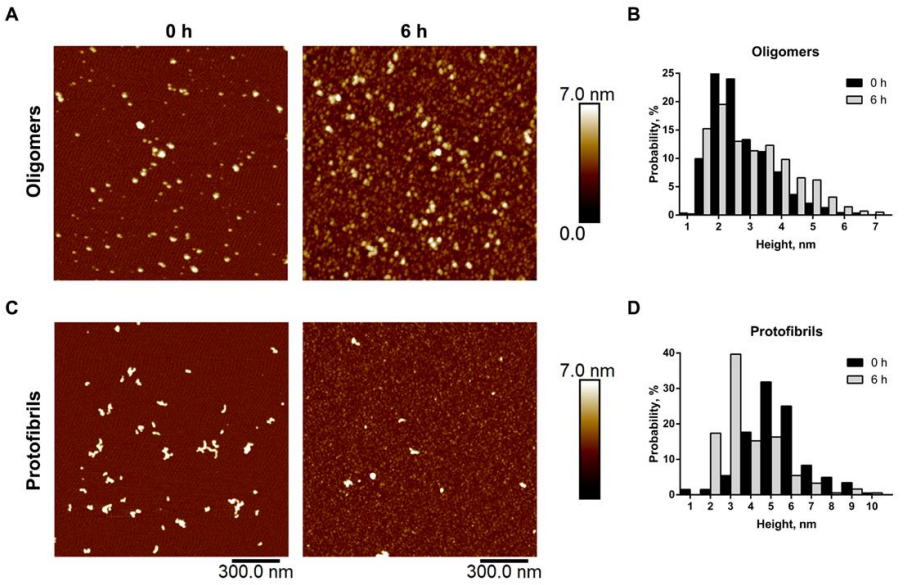

Figure 19. Aβ characterisation by atomic force microscopy. Representative images of Aβ oligomers and protofibrils prepared according to different protocols: (A) Oligomers – prepared using Protocol I; mainly 1-5 nm in size. (C) Protofibrils – prepared using Protocol II. (B) and (D) Histograms of height distribution of Aβ particles, n = 3 independent experiments. After preparation of Aβ oligomers and protofibrils they were diluted with cell culture medium to 2.5 μM and incubated for 6 h at 37 ºC under cell free conditions. After additional incubation of protofibrils, a higher fraction of small oligomers, about 1-2 nm in size was detected. In oligomers case the scanned surface was fully covered with small Aβ particles. The scanned size of the images is 1.5 x 1.5 μm. All images are represented in height scale from 0 to 7 nm.

The prepared Aβ oligomers and protofibrils itself did not induce inflammatory cytokine secretion in murine microglia. Neither TNF-α nor IL-1β release was detected after cell treatment with Aβ (Fig. 20). Therefore, we primed microglia cells with LPS to induce expression of inflammasome proteins. Then, we treated the cells with Aβ aggregates for 6 h. LPS induced TNF-α release, however, Aβ addition did not influence this cytokine secretion (Fig. 20B). We found that both Aβ oligomers and protofibrils triggered IL-1β release in microglia (Fig. 20A). Specific NLRP3 inflammasome inhibitor MCC950 completely blocked this effect.

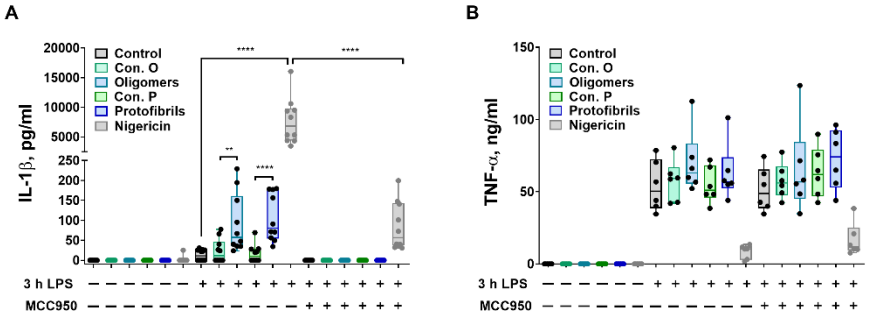

Figure 20. Aβ oligomers and protofibrils induce IL-1β release in murine microglia. Microglia were primed with 50 ng/ml LPS and treated with either Aβ oligomers or protofibrils for 6 h at 2.5 μM. Inhibitor MCC950 (1 μM) was added 20 min before the Aβ treatment. Nigericin (10 μM) was used as a positive control. Culture supernatants were collected and analysed by ELISA, for (A) IL-1β release, n = 10 and (B) TNF-α release, n = 6. Con O. – oligomer control, Con P. – protofibrils control. Data are represented using box-plots with data points showing independent experiments, **p < 0.01, ****p < 0.0001, one-way ANOVA followed by Bonferroni's multiple comparison test.

Then we assayed the treated cells for other components of inflammasome activation – caspase-1 and ASC specks. Both Aβ oligomers and protofibrils triggered ASC specks formation (Fig. 21). This suggests that these Aβ preparations can act as potent inducers of ASC speck formation.

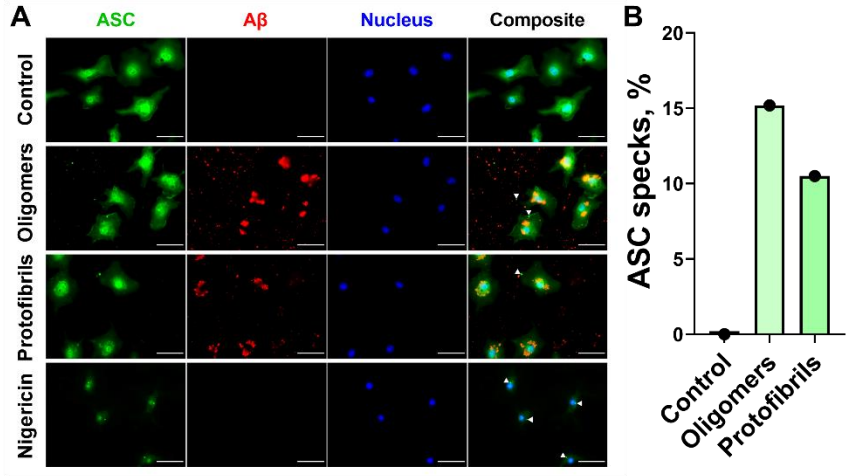

Figure 21. Aβ induced ASC speck formation. Microglia were primed with 50 ng/ml LPS and treated with either Aβ oligomers or protofibrils for 6 h at 2.5 μM concentration. Nigericin (10 μM) was used as a positive control. Cells

were immunostained with anti-Aβ (red), anti-ASC (green) and nuclear stain Hoechst (blue). a) Representative images of the staining. The images were taken using 60×-oil objective. The scale bars indicate 40 μm. White arrows show ASC specks. b) Quantification of ASC speck percent per cell, n = 1. In oligomers treatment, 59 cells were counted, in protofibrils – 57 cells were counted per treatment.

Aβ oligomers and protofibrils also induced caspase-1 cleavage to its active form, 20 kDa fragment called p20 (Fig. 22). Specific NLRP3 inflammasome inhibitor MCC950 reduced caspase-1 activation proving inflammasome activation by Aβ.

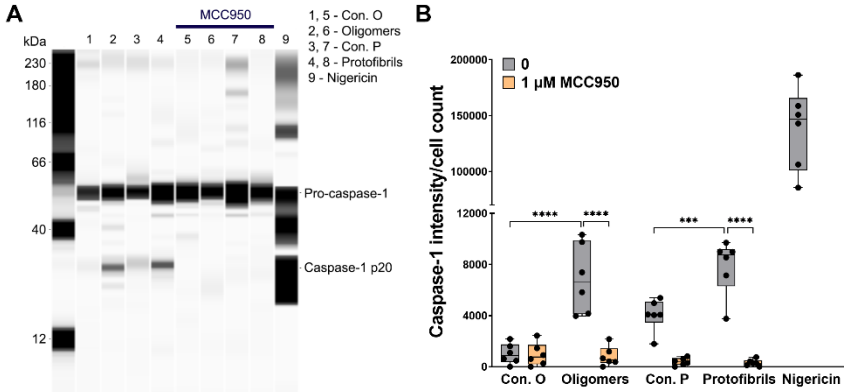

Figure 22. Aβ induced cleavage of caspase-1 in microglia cells. Microglia were primed with 50 ng/ml LPS and treated with either Aβ oligomers or protofibrils for 6 h at 2.5 μM concentration. Inflammasome inhibitor MCC950 (1 μM) was added 20 min before Aβ treatment. Nigericin (10 μM) was used as a positive control. Cleaved caspase-1 was determined in cell supernatants using western blot. (A) Representative image of western blot analysis. Con O. – oligomer control, Con P. – protofibrils control. (B) Caspase-1 p20 quantification. Caspase-1: 50 kDa – pro-caspase-1; 20 kDa – cleaved caspase-1 (p20), n = 6 independent experiments. Data are represented using box-plots with data points showing independent experiments, ***p < 0.001, ****p < 0.0001, one-way ANOVA followed by Bonferroni's multiple comparison test.

Generally, inflammasome activation outcome is cell death. However, we could not see a statistically significant decrease in the cell viability using metabolic activity assays: XTT assay (see method section) and assay for detection of LDH release (Fig. 23AB). Then we stained the nuclei with PI which enters only dead cells (Fig. 23CD). Again, we could not see a statistically significant cell death after the cell treatment with Aβ aggregates.

If we compare inflammasome activation level with Aβ aggregates to classical activator nigericin, we could see that only a small number of cells were activated (Fig. 23).

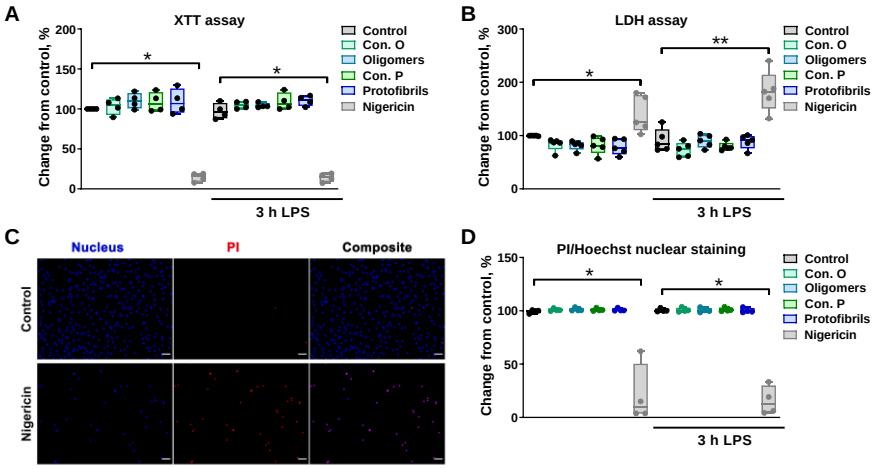

Figure 23. Aβ does not affect viability of microglia. Microglia were primed with 50 ng/ml LPS and treated with either Aβ oligomers or protofibrils for 6 h at a concentration of 2.5 μM. Nigericin (10 μM) was used as a positive control. To assess Aβ-induced cytotoxicity (A) Metabolic activity – XTT assay (n = 4 independent experiments), (B) LDH assay (n = 5 independent experiments), and (C, D) Propidium iodide (PI) and Hoechst nuclear staining (n = 4 independent experiments) were performed. (C) Representative images of nuclear staining; PI stains dead cells, Hoechst – all cells. The images were taken using 20× objective. The scale bars indicate 40 μm. (D) Quantification of PI and Hoechst nuclear staining. Con O. – oligomer control, Con P. – protofibrils control. Data are represented using box-plots with data points showing independent experiments, ****$p < 0.0001$, Kruskal–Wallis test in conjugation with a Dunn's post hoc test.

In order to evaluate the number of activated microglia cells, we analysed caspase-1 activation at a single-cell level using FLICA reagent. It contains FAM-YVAD-FMK – caspase-1 inhibitor probe, which covalently binds to only activated caspase-1. Again, only a small number of caspase-1 positive cells was detected (Fig. 24). We concluded that soluble Aβ aggregates activate inflammasome at a modest level and without a detectable amount of dead cells.

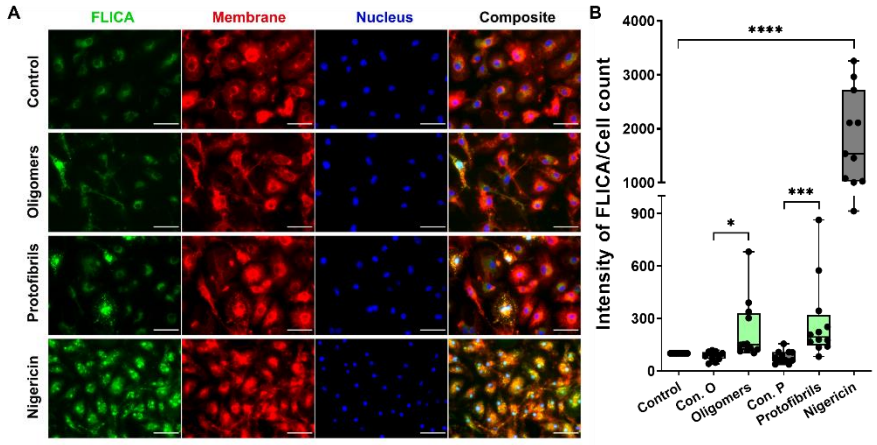

Figure 24. Aβ induced caspase-1 activation. Microglia were primed with 50 ng/ml LPS and treated with Aβ oligomers or protofibrils for 6 h at 2.5 μM concentration. Inflammasome inhibitor MCC950 (1 μM) was added 20 min before Aβ treatment. Nigericin (10 μM) was used as a positive control. Cells were stained for activated caspase-1 using fluorescently labelled inhibitor of caspase-1, FLICA (green), membrane stain (red) and nuclear stain Hoechst (blue). (A) Representative images of the staining. The images were taken using 60×-oil objective. The scale bars indicate 40 μm. (B) Caspase-1 quantification, n = 12 independent experiments. Con O. – oligomer control, Con P. – protofibrils control. Data are represented using box-plots with data points showing independent experiments, *p < 0.05 ***p < 0.001, Kruskal–Wallis test in conjugation with a Dunn's post hoc test.

One of inflammasome activation mechanism is related to phagocytosis. Other studies show that Aβ fibrils cause lysosomal damage and induce inflammasome activation (Halle *et al.*, 2008a; Sheedy *et al.*, 2013). Lysosomal damage can cause cathepsin release or further processes, like reactive oxygen species generation, and trigger the receptors of inflammasome. Therefore, we investigated the uptake of Aβ oligomers and protofibrils. Aβ was visualised by immunocytochemistry. CD68 was used as macrophage and lysosomal marker. Aβ signal co-localised with CD68 was analysed. Aβ oligomers and protofibrils were taken up by microglia (Fig. 25AC), however, there were no differences between these aggregates. Next, we investigated Aβ uptake by flow cytometry at a single cell level. We also found that Aβ is taken up by microglia (Fig. 25B) but a small population of cells, high in Aβ, was detected in both cases – oligomers and protofibrils (Fig. 25DE). We assume that this population may represent the cells which phagocytosed Aβ more efficiently.

This small population coincides with a low cellular response to Aβ compared to a strong activator nigericin having in mind inflammasome activation (caspase-1 activation and IL-1β release data). Therefore, the mechanism of inflammasome activation by Aβ oligomers might be related to the intensity of their phagocytosis.

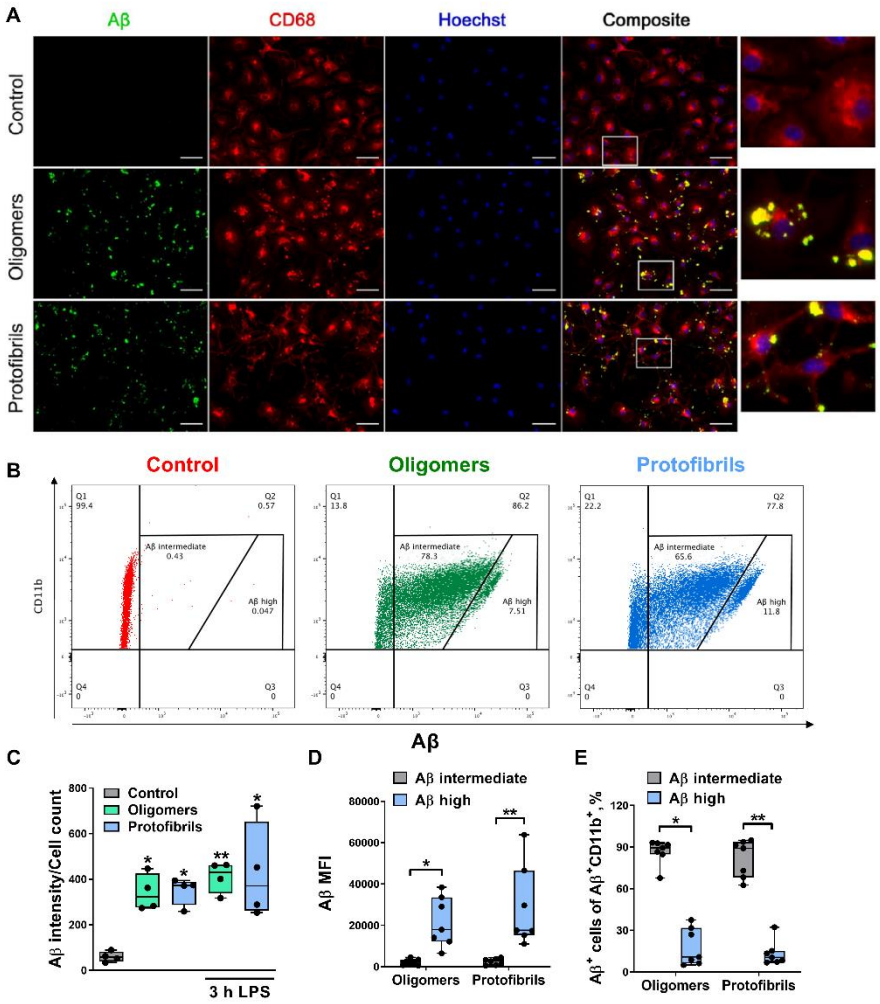

Figure 25. The uptake of Aβ oligomers and protofibrils by microglia. Microglia were primed with 50 ng/ml LPS and treated with either Aβ oligomers or protofibrils for 6 h at 2.5 μM concentration. Cells were immunostained with anti-Aβ (green), anti-CD68 – microglia and lysosomal marker (red), nuclear stain Hoechst (blue) and analysed by fluorescence microscope. (A) Representative images of one experiment. The images were taking using 40× objective. The scale bars indicate 40 μm. (C) Quantification of Aβ uptake – Aβ co-localization with CD68 was calculated, n = 4

independent experiments. Aβ uptake was also analysed by FACS. Cells were immunostained with anti-Aβ, and anti-CD11b was used to detect microglia. $CD11b^+A\beta^+$ cells were analysed. (B) Representative image of one experiment. (D) and (E) Quantification of Aβ uptake. $CD11b^+A\beta^+$ population highly rich in Aβ was referred as Aβ high. Other Aβ positive cells were referred as Aβ intermediate. (D) Fraction of $CD11b^+A\beta^+$ cells in Aβ intermediate and Aβ high populations, (E) MFI – median fluorescent intensity, n = 7 independent experiments. Data are represented using box-plots with data points showing independent experiments, *p < 0.05, **p < 0.01, ****p < 0.0001, Kruskal–Wallis test in conjugation with a Dunn's post hoc test for microscope analysis data, one-way ANOVA followed by Bonferroni's multiple comparison test for FACS analysis data.

3.2 Investigation of macrophage activation by polyomavirus VLPs

We showed that Aβ oligomers induce inflammasome activation in the microglia. Further, we explored the hypothesis that proteins of other origin, viral oligomeric proteins, could induce inflammation and activate inflammasome in macrophages. The model of human macrophages – THP-1 monocytes differentiated to macrophage-like cells (THP-1 macrophages) – was used as a cell model.

3.2.1. Characterisation of THP-1 macrophages

There are several protocols how to prepare macrophages from THP-1 monocytic cell line. We selected differentiation conditions according to (Chanput *et al.*, 2014). Firstly, we characterised THP-1 macrophage response to classical NLRP3 inflammasome activator. Nigericin induced cell death (Fig. 26A-C) and IL-1β release (Fig. 26D) in THP-1 macrophages. NLRP3 inflammasome inhibitor MCC950 significantly reduced these effects (Fig. 26E). In addition, MCC950 restored TNF-α secretion showing the inhibition of inflammasome.

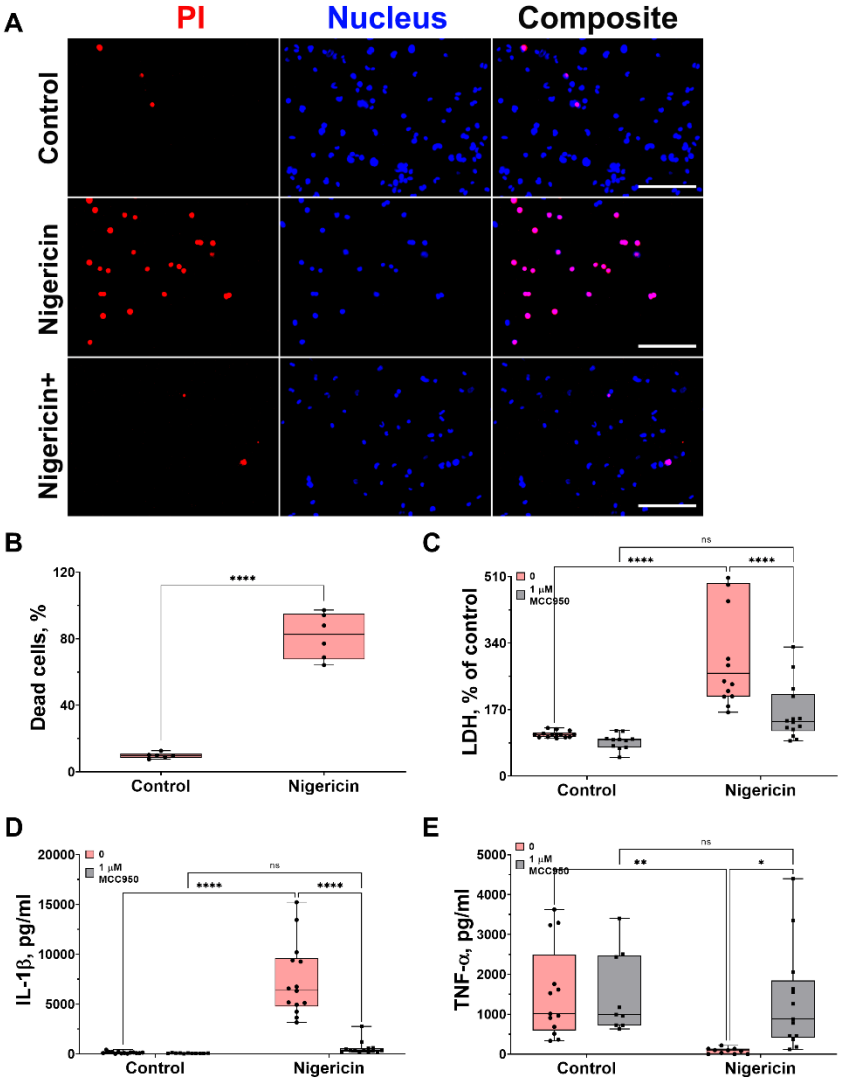

Figure 26. Nigericin induced cell death and IL-1β release in THP-1 macrophages. THP-1-ASC-GFP macrophages were treated for 24 h with nigericin (10 μM), inhibitor MCC950 (1 μM) was added 30 min before treatment. (A) PI (dead cells) and Hoechst (all cells) nuclear staining. The scale bars indicate 200 μm. "Nigericin+" – refers to Nigericin+MCC950. (B) Quantification of dead cells. (C) Cytotoxicity assessed by LDH assay. (D) IL-1β and (E) TNF-α secretion determined by ELISA. Data are represented using box plots, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA followed by Tukey's multiple comparison test.

Then, we used THP-1 cell line expressing ASC protein fused with GFP (THP-1-ASC-GFP) to observe ASC specks representing inflammasome assembly. We made time lapse experiment and found that after 3 h ASC pecks are formed and after 24 h the signal disappeared (Fig. 27). MCC950 inhibited ASC speck formation.

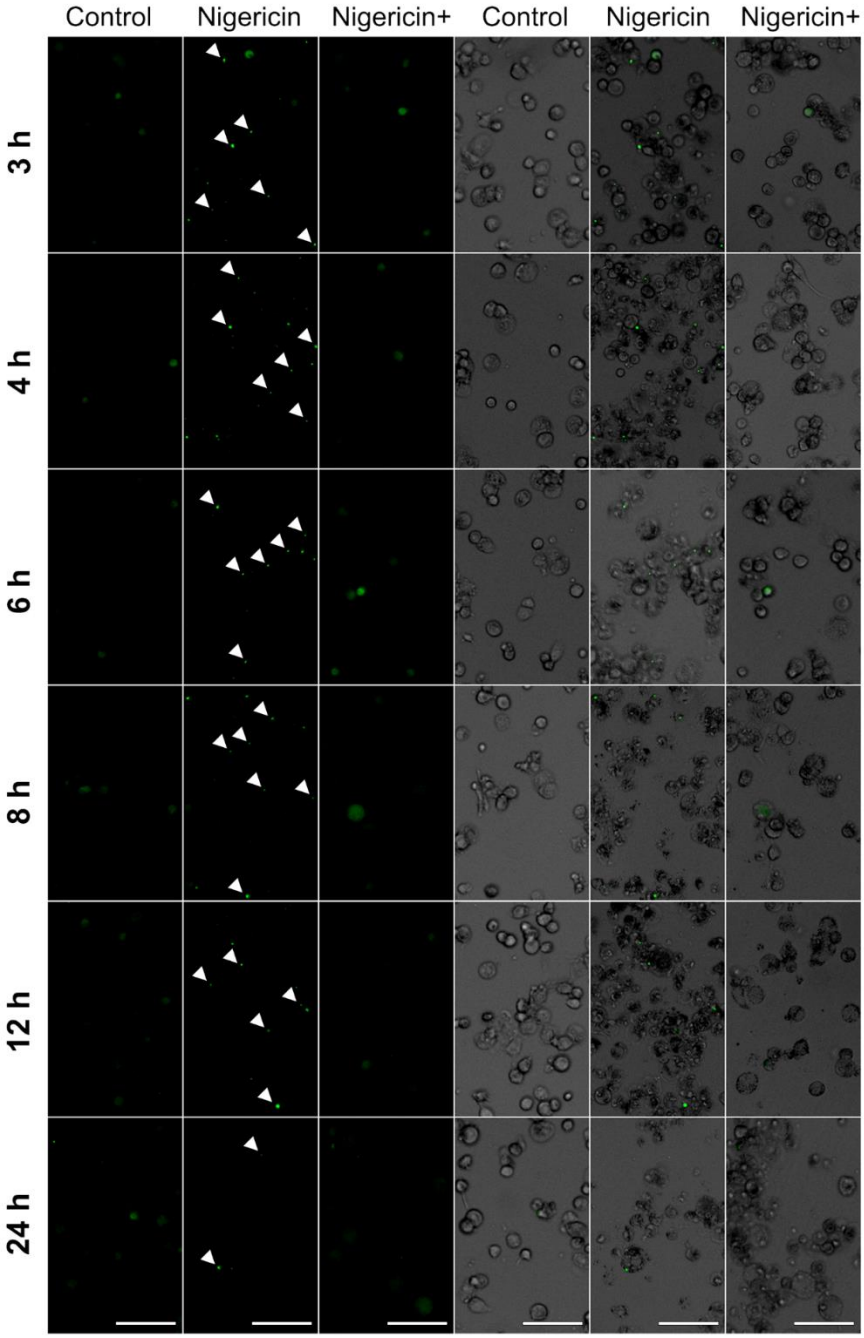

Figure 27. Time lapse of ASC speck formation after nigericin treatment in THP-1 macrophages. THP-1-ASC-GFP macrophages were treated for 3-24 h with nigericin (10 μM), inhibitor MCC950 (1 μM) was added 30 min before the treatment. Formation of ASC specks (dots) was visualised by fluorescent microscope. Arrows show ASC specks. "Nigericin+" – refers to Nigericin+MCC950. The scale bars indicate 50 μm. Representative images of one experiment are shown, N = 1.

Next, we assayed activated caspase-1 using FLICA assay. Caspase-1 is secreted from activated macrophages, thus, we analysed after 1 h treatment with nigericin. We detected activated caspase-1 in the cells (Fig. 28). Therefore, we found all inflammasome activation components in THP-1 macrophages.

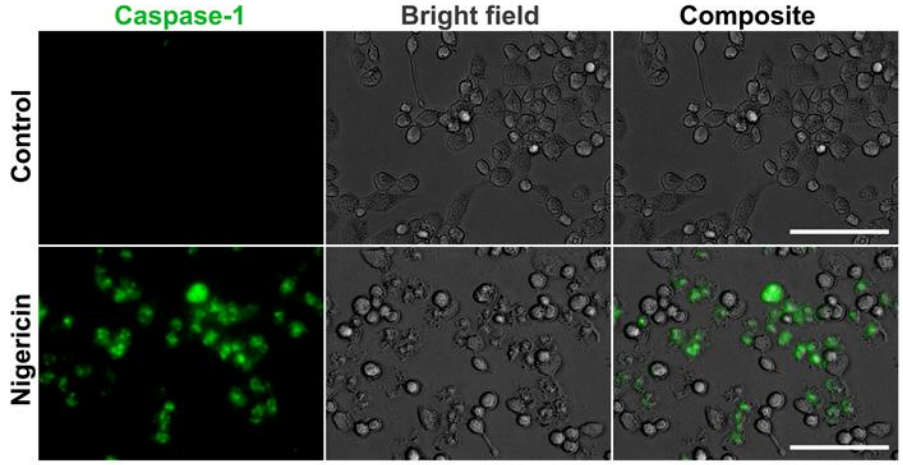

Figure 28. Activated caspase-1 detection after nigericin treatment in THP-1 macrophages. Macrophages were treated for 1 h with with nigericin (10 μM). Activated caspase-1 was determined inside the cells. Representative images of the caspase-1 staining by FLICA (green) reagent after 1 h treatment with nigericin. The scale bars indicate 100 μm. N = 1.

We investigated viral oligomeric proteins of diverse structure to explore how structural properties of virus-derived particles can determine cell activation outcome. The models of viral antigens were these recombinant oligomeric viral particles: oligomers of filamentous structure composed of nucleocapsid proteins of measles and mumps viruses; spherical particles of VP1 proteins of KI and MC polyomaviruses (PyV). Recombinant viral N proteins form long rod-shaped structures, about 20 nm in diameter, similar to the nucleocapsids of native viruses infecting cells, called nucleocapsid-like particles (NLPs) (Samuel *et al.*, 2002; Slibinskas *et al.*, 2004). Particles of

KIPyV (heterogeneous size, 20-60 nm in diameter) and MCPyV (homogeneous size, 45-50 nm in diameter) are composed of VP1 pentamers which self-assembly into spheres similar to the capsids of native PyV, called virus-like particles (VLPs).

The first cell activation step is cell interaction with an activating particle. Therefore, we started from the uptake assay of viral oligomeric proteins by THP-1 macrophages. Viral proteins were detected by immunocytochemistry. We also stained the cells for macrophage and lysosomal marker CD68. The uptake of all investigated proteins according to the co-localisation of CD68 and viral proteins was clearly seen, even vesicle-like structures were detected in all stains (Fig. 29). Thus, all investigated viral proteins, VLPs and NLPs, were detected and taken up by THP-1 macrophages.

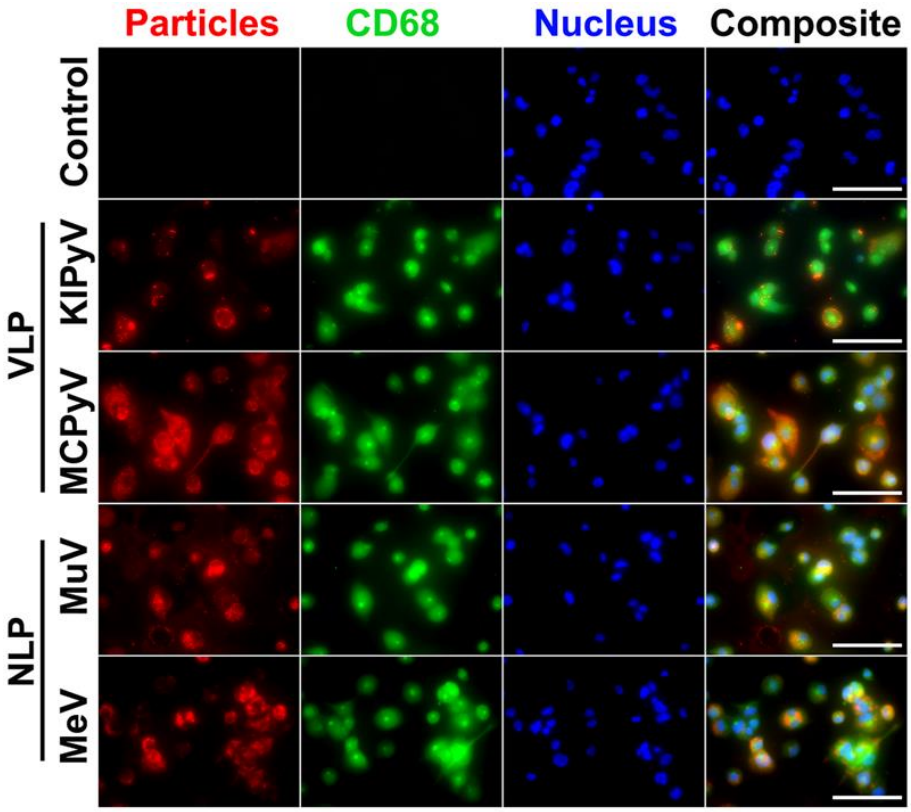

Figure 29. NLPs of measles and mumps viruses, and PyV-derived VLPs were taken up by THP-1 macrophages. Macrophages were treated with recombinant viral proteins for 24 h at 20 μg/ml concentration. Cells were immunostained with anti-NLP and anti-VLP monoclonal antibodies (red), anti-CD68 – macrophage and lysosomal marker (green), nuclear stain Hoechst33342 (blue) and analysed by fluorescence microscopy. The negative

control – secondary antibody alone is referred as a control. Images were taken using 40× objective. The scale bars indicate 100 μm. All experiments were performed in triplicate. Representative images of one experiment are shown.

3.2.2. NLPs did not activate THP-1 macrophages

We investigated if viral oligomeric proteins could induce inflammatory response. We started from filamentous viral oligomeric proteins – N proteins of measles and mumps viruses. We treated THP-1 macrophages with these recombinant proteins forming NLPs and assayed for inflammatory cytokines IL-1β and TNF-α secretion, and cell death. However, we did not observe any cellular response (Fig. 30) and cell viability did not change.

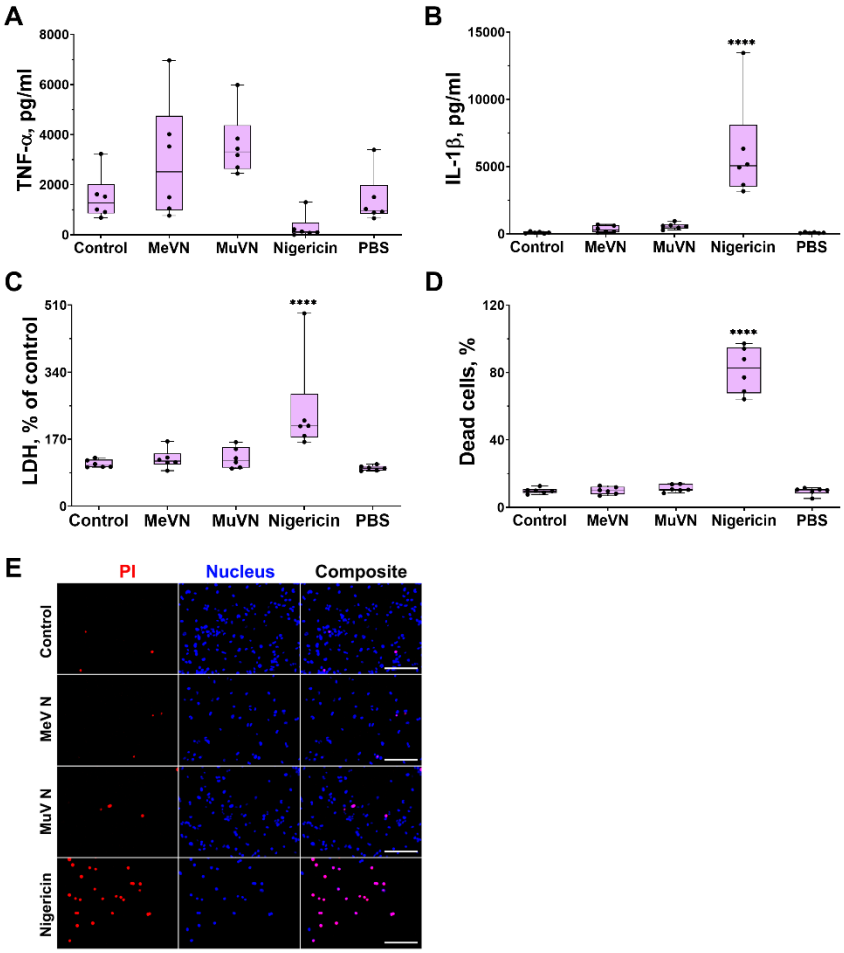

Figure 30. The NLPs of measles and mumps viruses did not induce inflammatory response in human THP-1 macrophages. THP-1 monocytes

differentiated to macrophages were treated with recombinant NLPs of measles and mumps viruses for 24 h at a concentration of 20 μg/ml. Nigericin (10 μM) was used as a positive control. Supernatants were collected and analysed by ELISA, for (A) TNF-α and (B) IL-1β release. To assess the cytotoxicity, (C) LDH assay and (D) PI and Hoechst nuclear staining were performed. PI indicates dead cells and Hoechst stains nuclei of all cells. Graph shows quantification of dead cells. (E) Representation images of nuclear staining. The images were taken using 20× objective. The scale bars indicate 200 μm. Data are represented using box-plots with data points showing independent experiments, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA followed by Tukey's multiple comparison test. Unless otherwise specified, stars show statistically significant results compared to control.

We investigated the influence of Aβ oligomers on cell activation using mouse model – primary microglia. Human and mouse models could show different cellular response, thus, we activated primary murine microglia with recombinant NLPs as we did with THP-1 macrophages. We treated microglia cells with NLPs with and without LPS pre-stimulation, however, these protein oligomers had no influence on cell activation (Fig. 31). We detected the uptake of N proteins (Fig. 31D) but no change in LDH, IL-1β, TNF-α release (Fig. 31ABC). We concluded that recombinant filamentous proteins are not able to activate macrophages and trigger inflammatory response in both mouse and human cell models. In further experiments, we used only human cell model.

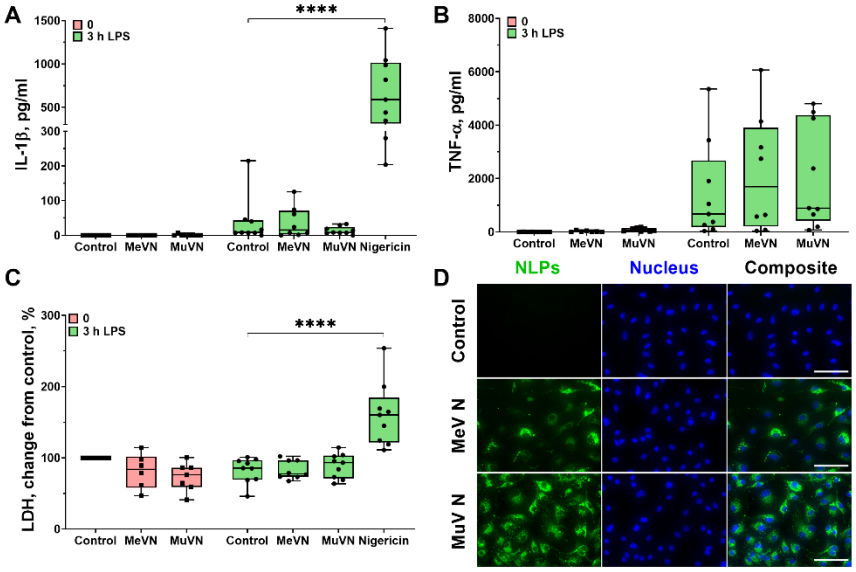

Figure 31. The NLPs of measles and mumps viruses did not induce inflammatory response in murine microglia. Microglia were primed with 50 ng/ml LPS and treated with recombinant NLPs derived from measles and mumps viruses for 24 h at a concentration of 20 μg/ml. Nigericin (10 μM) was used as a positive control. Supernatants were collected and analysed by ELISA, for (A) TNF-α and (B) IL-1β release. To assess the cytotoxicity, (C) LDH assay was performed. (D) Cells were immunostained with anti-NLP monoclonal antibodies (green), nuclear stain Hoechst33342 (blue) and analysed by fluorescence microscopy. The negative control – secondary antibody alone is referred as a control. Images were taken using 40× objective. The scale bars indicate 100 μm. Representative images of one experiment are shown. Data are represented using box-plots with data points showing independent experiments, n = 9, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA followed by Tukey's multiple comparison test. Unless otherwise specified, stars show statistically significant results compared to control.

3.2.3. PyV-derived VLPs induced inflammatory response in THP-1 macrophages

Next, we investigated cell activation pattern after treatment with PyV-derived recombinant VLPs forming spherical structures. We used these VLPs of human PyV: KIPyV (heterogeneous structures, 20-60 nm in diameter) and MCPyV (homogeneous structures, 45-50 nm in diameter). They are composed of VP1 proteins that form pentamers self-assembling into spherical particles. We activated THP-1 macrophages with these particles and investigated whether they induced inflammatory response. Both KIPyV and MCPyV VLPs provoked a release of inflammatory cytokines IL-6 and TNF-α (Fig. 32AB). Larger size MCPyV VLPs induced a significantly higher TNF-α secretion. We also assayed cell cultures for anti-inflammatory cytokine IL-10 release, however, we have not seen any secretion of this cytokine (data not shown). Therefore, in contrast to filamentous NLPs of measles and mumps viruses, spherical PyV-derived VLPs induced the inflammatory response in THP-1 macrophages.

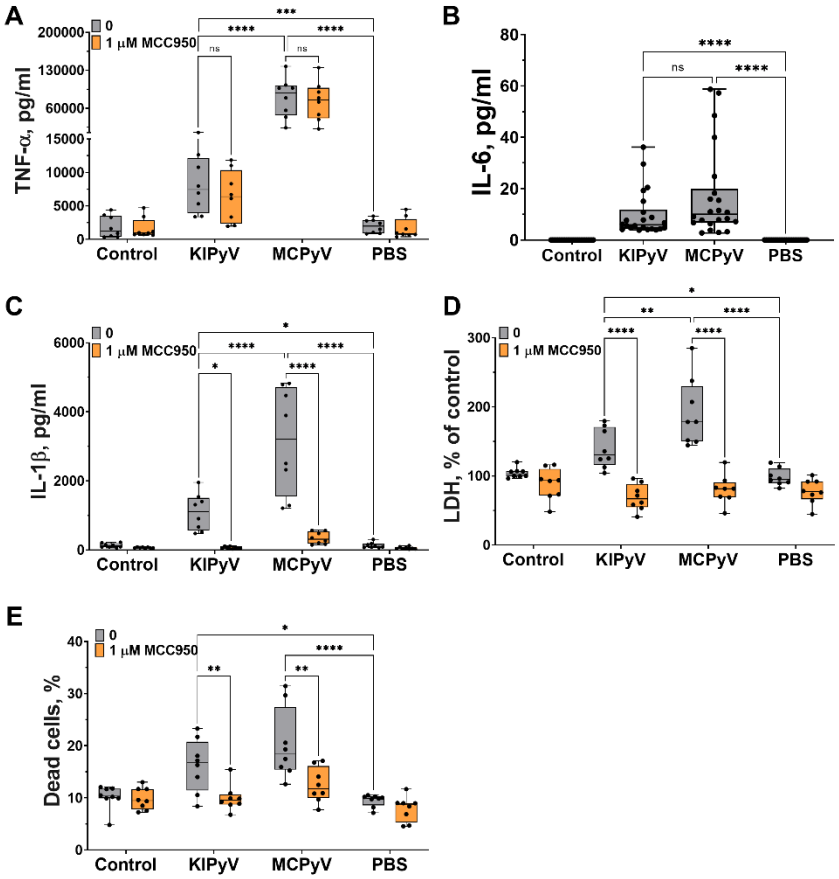

Figure 32. PyV-derived VLPs induced release of inflammatory cytokines TNF-α and IL-6 and activated NLRP3 inflammasome in human THP-1 macrophages. THP-1 monocytes differentiated to macrophages were treated with recombinant PyV-derived VLPs for 24 h at a concentration of 20 μg/ml. MCC950 (1 μM) was added 30 min before treatment with VLPs. Nigericin (10 μM) was used as a positive control. Supernatants were collected and analysed by ELISA for (A) TNF-α, (B) IL-6, and (C) IL-1β secretion. To assess the cytotoxicity, (D) LDH assay and (E) PI and Hoechst nuclear staining were performed. Graph shows quantification of dead cells (PI indicates dead cells and Hoechst stains nuclei of all cells). Data are represented using box-plots with data points showing independent experiments, n = 8, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA followed by Tukey's multiple comparison test. Unless otherwise specified, stars show statistically significant results compared to control.

Then, we tested NLRP3 inflammasome activation in THP-1 macrophages after their treatment with PyV-derived VLPs. KIPyV and MCPyV VLPs induced IL-β release (Fig. 32C) followed by the cell death according to LDH release assay (Fig. 32D) and nuclear staining of dead cells with PI assay (Fig. 32E). Specific NLRP3 inflammasome inhibitor MCC950 completely blocked these effects proving NLRP3 inflammasome activation by PyV VLPs. In addition, MCPyV VLPs provoked higher cell activation as compared to KIPyV VLPs.

NLRP3 inflammasome consists of an adaptor protein ASC. Aggregates of ASC monomers also can accumulate in the cells or be released outside the cells. We used THP-1 cell line expressing ASC protein fused with GFP (THP-1-ASC-GFP) to observe the inflammasome activation. PyV VLPs induced ASC speck formation in THP-1-ASC-GFP (Fig. 33). MCPyV VLPs triggered apparently higher ASC speck number compared to KIPyV VLPs, however, it was not statistically significant. In addition, we could not discriminate which aggregates of ASC-GFP protein are a part of the inflammasome and which ones are present in the cells as individual ASC specks. NLRP3 inflammasome inhibitor MCC950 significantly reduced ASC specks detected after VLPs treatment (Fig. 33B), therefore, we confirmed inflammasome formation in PyV VLP-treated cells. Furthermore, expression of ASC protein is induced by the activation of NFκ-β signalling pathway (Hoss *et al.*, 2017). As THP-1 cells turned green after PyV VLP treatment compared to the control (Fig. 33A), we also demonstrated NFκ-β activation by these VLPs. In addition, expression of ASC protein was not reduced by MCC950 inhibitor as a green colour indicates the presence of ASC protein in the cells (Fig. 33A).

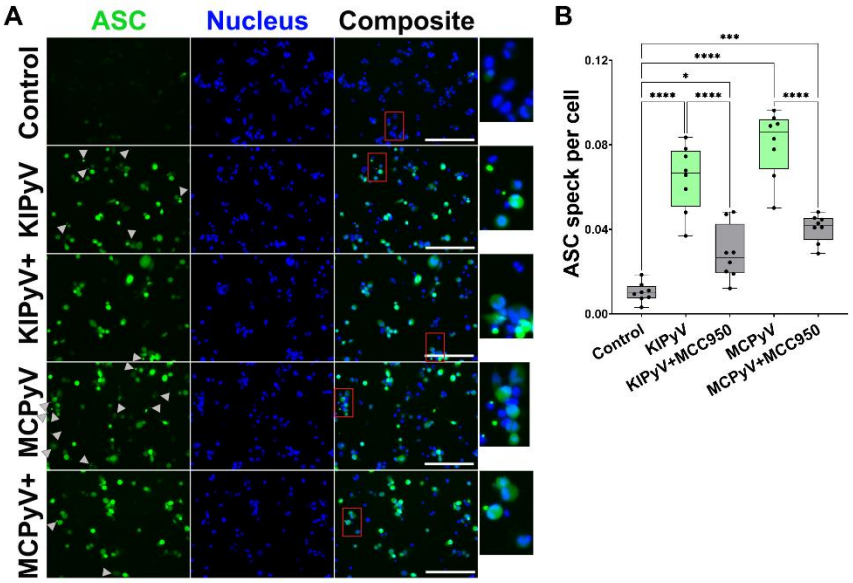

Figure 33. Recombinant PyV-derived VLPs induced ASC speck formation in the human THP-1 macrophages. Transformed THP-1 monocytes expressing ASC protein fused to GFP were differentiated to macrophages and treated with recombinant PyV-derived VLPs for 24 h at a concentration of 20 μg/ml. Inhibitor MCC950 (1 μM) was added 30 min before VLP treatment. After the treatment, formation of ASC specks was visualised by fluorescent microscope. (A) Representative images of one experiment. White arrows show ASC specks. The images were taken using 20× objective. The scale bars indicate 200 μm. (B) Quantification of ASC speck count per cell. Data are represented using box-plots with data points showing independent experiments, n= 8, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, one-way ANOVA followed by Tukey's multiple comparison test. Unless otherwise specified, stars show statistically significant results compared to control.

Next, we made time-lapse experiments to identify when cell activation by PyV VLPs begins. We took images of THP-1-ASC-GFP after 3-24 h treatment with PyV VLPs. We found that the cells obviously became green with expressed ASC-GFP protein in 12 h (Fig. 34). After 24 h treatment the cells were strongly activated and had ASC specks.

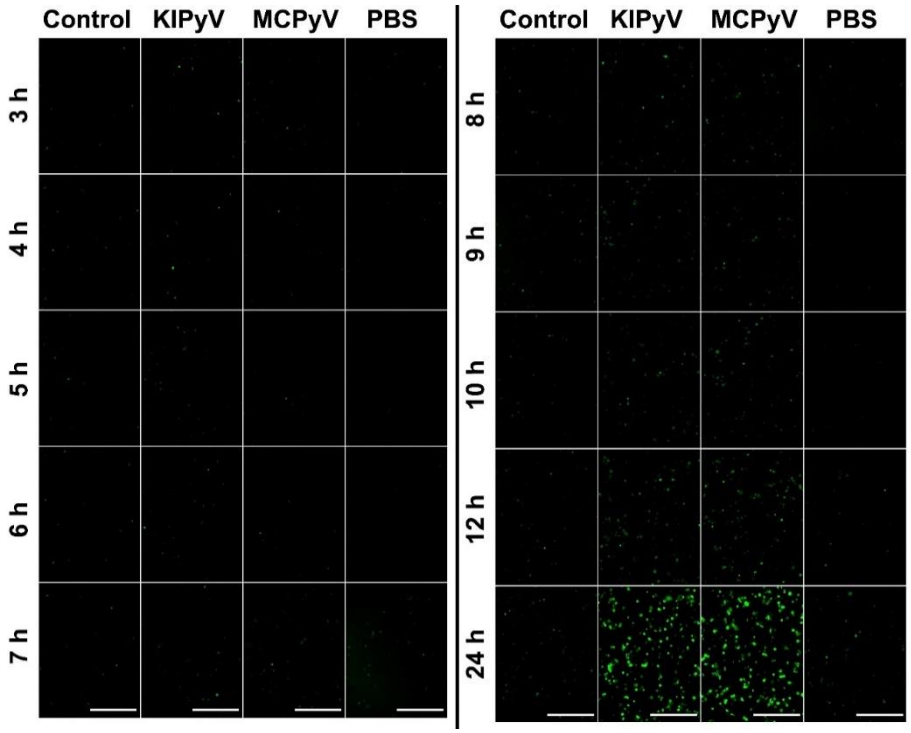

Figure 34. Time lapse of ASC speck formation induced by PyV-derived VLPs in human THP-1 macrophages. Transformed THP-1 monocytes expressing ASC protein fused to GFP were differentiated to macrophages and treated with recombinant PyV-derived VLPs for 3-24 h at a concentration of 20 μg/ml. Cell activation was monitored at different time points using ASC speck formation assay. ASC specks were visualised by fluorescent microscope. Representative images of one experiment are shown. The images were taken using 20× objective. The scale bars indicate 200 μm. N = 1.

Then, we made another experiment starting time lapse experiment after 14 hrs treatment. We did not find considerable differences in cells treated for14-24 h (Fig. 35B). All cells were more or less of the same green intensity. In addition, we also made an experiment with wild-type THP-1 measuring LDH release. Again, there were no differences in 15-23 hrs treatment with PyV VLPs (Fig 35A). We concluded that activation of THP-1 macrophages begins at about 12 h after PyV VLPs treatment and stays steady from about 15 h time point.

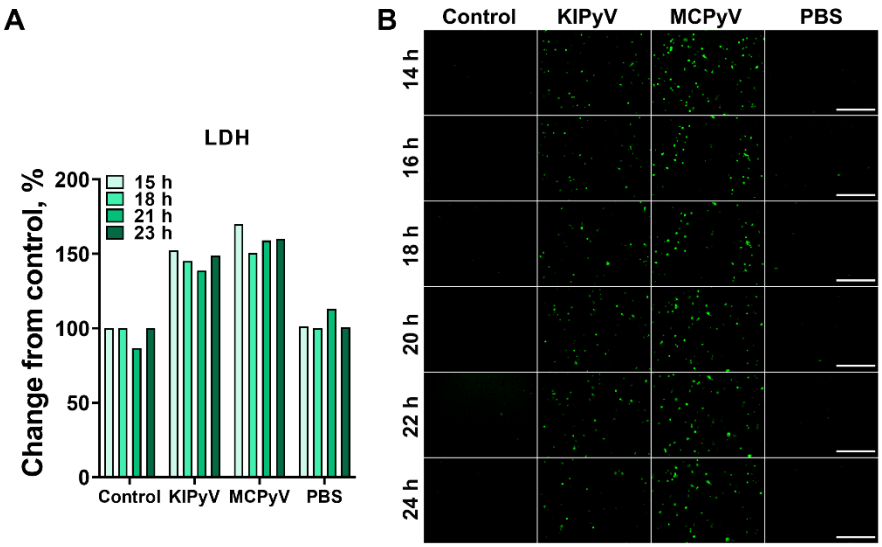

Figure 35. Time lapse of PyV VLP-induced activation of THP-1 macrophages. Wild-type and transformed THP-1 monocytes expressing ASC protein fused to GFP were differentiated to macrophages and treated with recombinant PyV-derived VLPs for 14-24 h at a concentration of 20 µg/ml. Cell activation was monitored at different time points using LDH and ASC speck formation assays. (A) LDH assay was performed on cell culture supernatant. The formation of ASC specks was visualised by fluorescent microscope. (B) Representative images of one experiment. The images were taken using 20× objective. The scale bars indicate 200 µm. N = 1.

During inflammasome activation process, activated caspase-1 cleaves immature IL-1β to its active form. Therefore, we tested the cells for p20 fragment of activated caspase-1. Having the time lapse experiment data we collected cell culture supernatant for caspase-1 assay after 15 h – from the steady cell activation time point in order not to lose the active enzyme. We could see p20 fragment after PyV VLP treatment. Moreover, MCC950 inhibited generation of the cleaved caspase-1 form proving inflammasome activation (Fig. 36A). Then, we investigated caspase-1 activation at a single cell level using FLICA reagent. It contains FAM-YVAD-FMK – caspase-1 inhibitor probe, which covalently binds to only activated caspase-1. We found a significant increase in caspase-1 positive cells after the cell treatment with PyV VLPs (Fig. 36BC). Again, MCC950 inhibited caspase-1 activation. In addition, we detected only a small percent of activated cells (Fig. 36C).

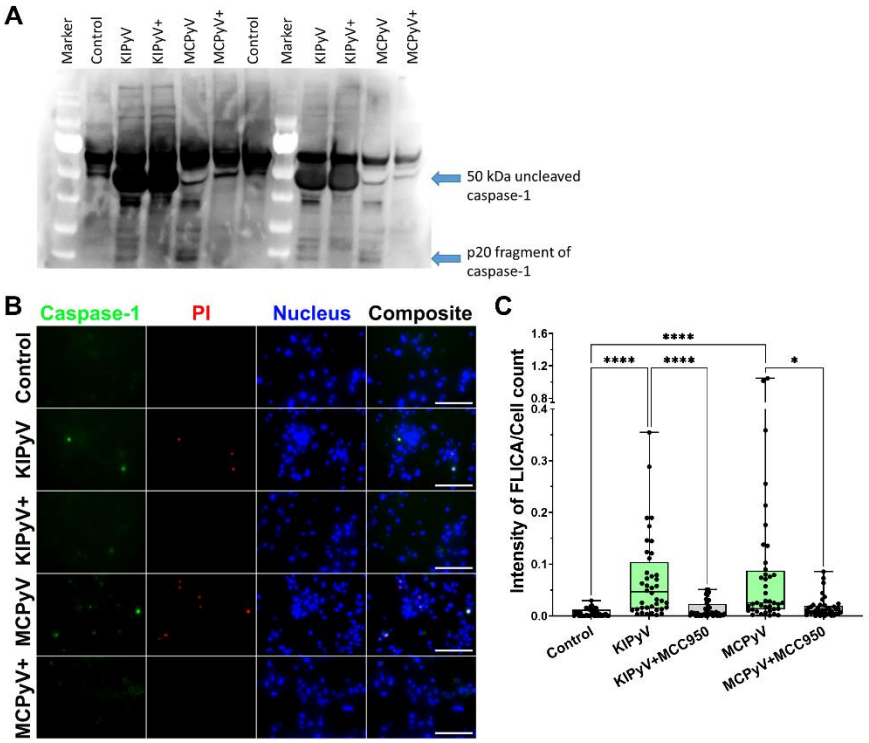

Figure 36. PyV-derived VLPs induced caspase-1 activation in human THP-1 macrophages. THP-1 monocytes differentiated to macrophages were treated with recombinant PyV-derived VLPs for 15 h at a concentration of 20 μg/ml. Inflammasome inhibitor MCC950 (1 μM) was added 30 min before VLP treatment. (A) Cleaved caspase-1 was determined in cell supernatants using WB. Caspase-1: 50 kDa – pro-caspase-1; 20 kDa – cleaved caspase-1 (p20). "+" – refers to MCC950 pre-treatment. Duplicates of one experiment are shown. (B) Cells were stained for activated caspase-1 using fluorescently labelled inhibitor of caspase-1 (FLICA, green), dead cell nuclear stain PI (red) and nuclear stain Hoechst (blue). The images were taken using 40× objective. Representative images of the staining are shown. The scale bars indicate 100 μm. (C) Caspase-1 quantification according to FLICA analysis, n = 40 photos per condition. N = 1 independent experiment. Data are represented using box-plots with individual data points, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, Kruskal–Wallis test with Dunn's post hoc.

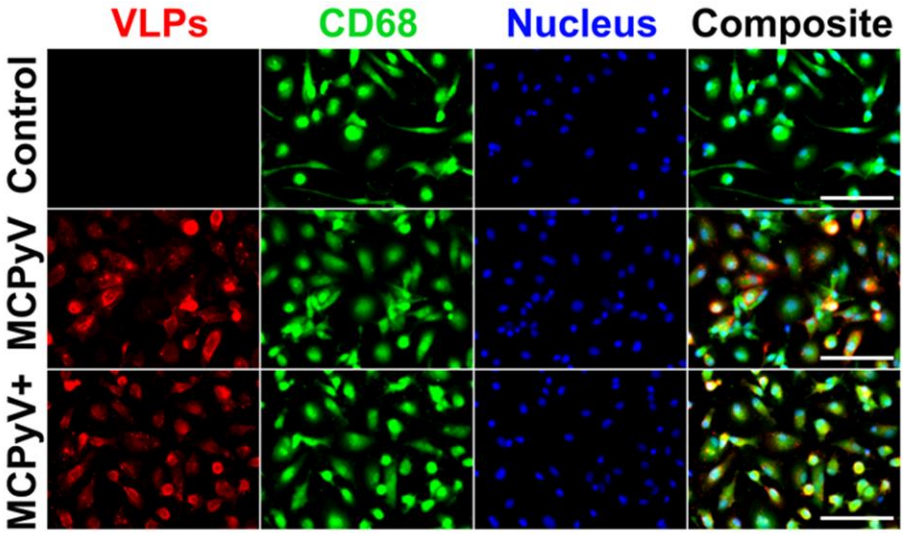

Figure 37. PyV-derived VLPs were taken up by human primary macrophages. (a) Human macrophages derived from peripheral blood mononuclear cells were treated with recombinant viral proteins for 24 h at 20 μg/ml concentration. Cells were immunostained with anti-VLP monoclonal antibodies (red), anti-CD68 – macrophage and lysosomal marker (green), nuclear stain Hoechst33342 (blue) and analysed by fluorescence microscopy. The negative control – secondary antibody alone is referred as a control. Images were taken using 40× objective. The scale bars indicate 100 μm.

Finally, to prove inflammatory response induced by human PyV VLPs we used primary human macrophages derived from monocytes of peripheral blood mononuclear cells, taking into account that gene mutations in cell lines can sometimes alter cellular response to activating agents. First, we analysed the uptake of KIPyV and MCPyV VLPs. These particles were taken up both by primary human macrophages (Fig. 37) and by THP-1 macrophages (Fig. 29). After cell treatment with MCPyV VLPs we detected caspase-1 activation according to FLICA assay (Fig. 38AC). Caspase-1 positive cells were dead (PI staining) demonstrating pyroptotic cell death (Fig. 38AB). NLRP3 inflammasome inhibitor MCC950 significantly reduced caspase-1 activation. Similarly to THP-1 macrophages, a small number of primary macrophages were activated according to the number of caspase-1 positive and dead cells. In addition, a significant increase in TNF-α and IL-1β release (Fig. 38DE) was detected after MCPyV VLP treatment. Therefore, activation of primary human macrophages with VLPs revealed inflammatory response induced by PyV VLPs.

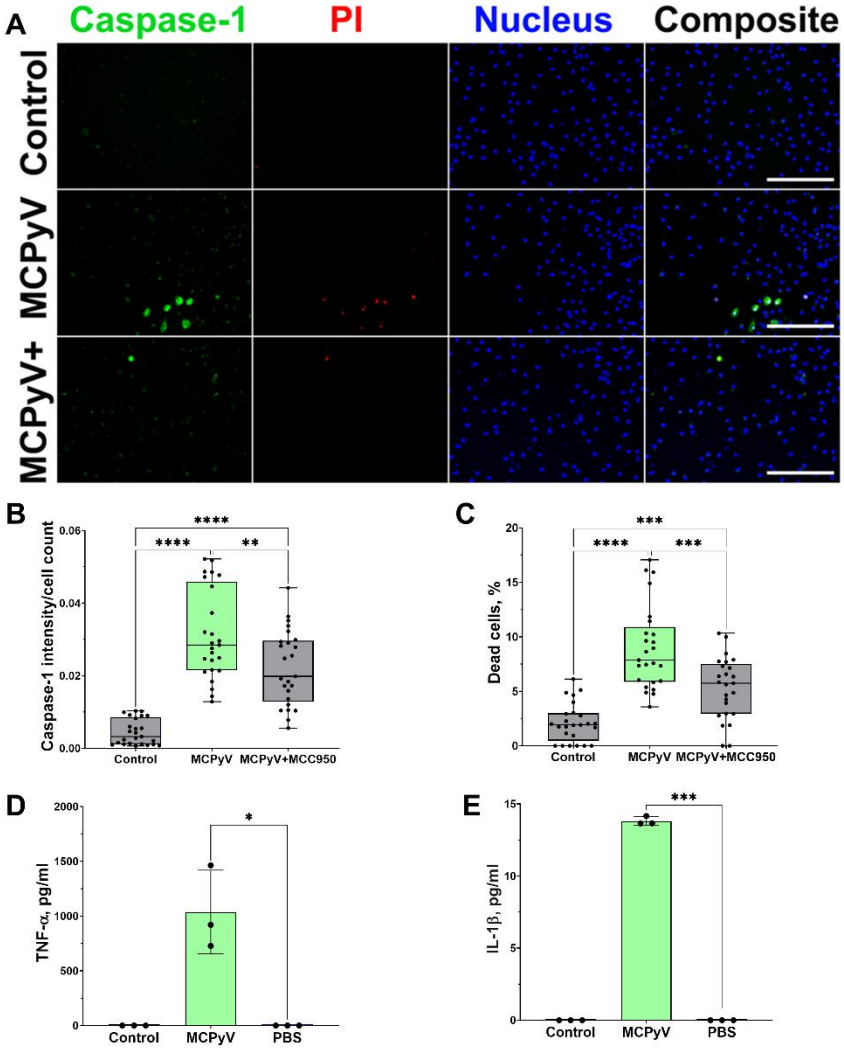

Figure 38. PyV VLPs activate the inflammasome in primary human macrophages. Human macrophages derived from peripheral blood mononuclear cells were treated with recombinant PyV-derived VLPs for 15 or 24 h at a concentration of 20 μg/ml. Inhibitor MCC950 (1 μM) was added 30 min before the treatment with VLPs. (A) Cells were stained for activated caspase-1 using FLICA (green), dead cell nuclear stain PI (red) and nuclear stain Hoechst (blue) after 15 h. The images were taken using 20× objective. Representative images of the staining are shown. The scale bars indicate 100 μm. MCPyV+ means MCPyV+MCC950. (B) Quantification of dead cells, n = 25 photos per condition. (C) Caspase-1 quantification according to FLICA analysis, n = 25 photos per condition. Data are represented box-plots with individual data points. Culture supernatants collected after 24 h were analysed

by ELISA for (D) TNF-α and (E) IL-1β release, n = 3 technical repeats. N = 1 independent experiment. Data are represented using bar graph with dots showing individual data points, *p < 0,05, **p < 0,01, ***p < 0,001, ****p < 0,0001, one-way ANOVA followed by Tukey's multiple comparison test. Unless otherwise specified, stars show statistically significant results compared to control.

In summary, VLPs induced NFκ-β signalling pathway and provoked inflammasome assembly following the secretion of inflammatory cytokines and cell death. We also showed that VLPs of different size promote different strength of cell activation signal. Heterogeneous structure KIPyV induced lower cell activation than homogeneous large-sized MCPyV. It could be that small VLPs did not activate macrophages or evoked only a modest cellular response.

3.2.4. VLP-induced inflammasome activation is related to lysosomal damage

We further investigated the mechanism of inflammasome activation by PyV-derived VLPs. It is likely that accumulation of proteins in lysosomes can damage them and induce the release of cathepsins, like cathepsin B. Cathepsin B is one of the NLRP3 inflammasome activators. We could down-regulate IL-1β release by cathepsin B inhibitor CA-074 Me (Fig. 39A). However, CA-074 Me did not reduce cell death (Fig. 39BC). We increased CA-074 Me concentration, however, it still did not reduce PyV VLPs induced cell death (Fig. 39DE).

Then we blocked cell death using K777 which is a broad-spectrum inhibitor of cathepsins. We could see a decrease in the cell death (Fig. 39FG). In addition, K777 significantly reduced IL-1β release showing the role of cathepsins in PyV VLP-induced inflammasome activation (Fig. 39H). However, IL-1β level did not drop to control baseline. There are controversial published data about particle-induced inflammasome activation. Some of the previous studies show that nanoparticles induce NLRP3 inflammasome activation via phagosomal destabilisation (Hornung *et al.*, 2008). Other research demonstrates that different nanoparticles trigger different effect depending on the composition and structure of the particle (Rashidi *et al.*, 2020). For example, cholesterol crystals activate NLRP3 inflammasome, however, inhibitors of cathepsins reduce only IL-1β release and do not change cell death. A similar effect we could see in our study – the inhibitor of cathepsin B did not reduce cell death after VLP treatment.

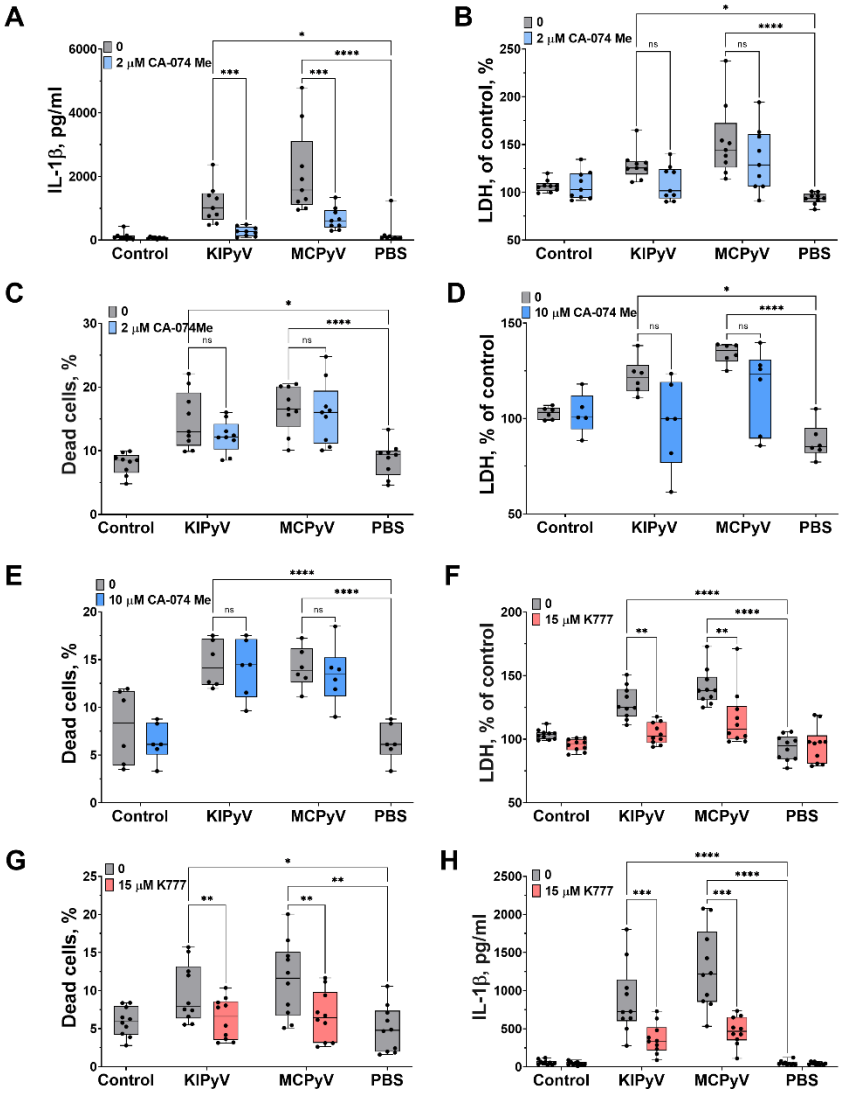

Figure 39. Cathepsin B inhibitor reduced VLP-induced IL-1β release while pan-cathepsin inhibitor (K777) decreased both IL-1β release and cell death in human THP-1 macrophages. THP-1 monocytes differentiated to macrophages were treated with recombinant PyV-derived VLPs for 24 h at a concentration of 20 μg/ml. Inhibitors CA-074 Me (2 or 10 μM) and K777 (15 μM) were added 30 min before VLP treatment. Supernatants were collected and analysed by ELISA for (A, H) IL-1β release. To assess cytotoxicity (B, D, F) LDH assay, and (C, E, G) PI and Hoechst nuclear staining were performed. PI indicates dead cells and Hoechst stains nuclei of all cells. Data are represented using box-plots with data points showing

independent experiments, n = 6-10, *p < 0,05, **p < 0,01, ***p < 0,001, ****p < 0,0001, one-way ANOVA followed by Tukey's multiple comparison test. Unless otherwise specified, stars show statistically significant results compared to control.

3.2.5. VLPs mediated inflammasome activation via K+ efflux

It is assumed that inflammasome can be activated by further processes followed by lysosomal damage, for example, reactive oxygen species and K^+ ion efflux. On the other hand, cell death due to membrane damage could be an irreversible process. The role of cathepsins in inflammasome activation is not fully understood. For example, it was shown that cathepsins can mediate activation of pore forming protein gasdermin D. (Selkrig *et al.*, 2020). Therefore, we assumed that PyV VLPs induced inflammasome activation via lysosomal damage, however, further investigations of mechanisms related to phagocytosis-induced cell death are required.

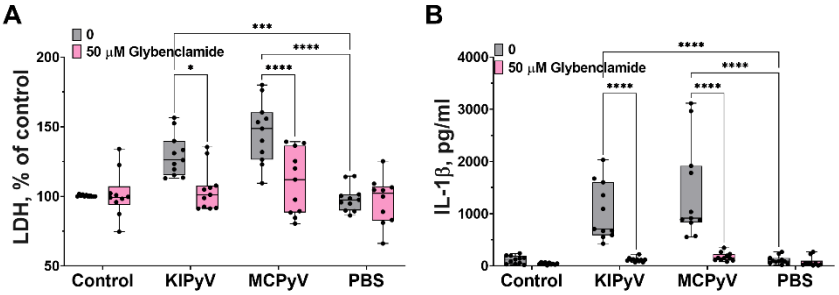

Figure 40. K+ ion efflux inhibitor (glybenclamide) reduced PyV VLP-induced cell death and IL-1β release in human THP-1 macrophages. THP-1 monocytes differentiated to macrophages were treated with recombinant PyV-derived VLPs for 24 h at a concentration of 20 μg/ml. Inhibitor glybenclamide (50 μM) was added 30 min before VLP treatment. To assess the cytotoxicity, (A) LDH assay was performed. Collected culture supernatants were analysed by ELISA for (B) IL-1β release. Data are represented using box-plots with data points showing independent, n = 11, *p < 0,05, **p < 0,01, ***p < 0,001, ****p < 0,0001, one-way ANOVA followed by Tukey's multiple comparison test. Unless otherwise specified, stars show statistically significant results compared to control.

Next, we tested the hypothesis that VLP-induced cell death can be associated with others than cathepsin-related inflammasome activation mechanisms. We inhibited K^+ ion efflux using an inhibitor called

glybenclamide or glyburide. It significantly reduced PyV VLP-induced cell activation (Fig. 40). As with K777, we could see only partial inhibition of IL-1β release (Fig. 40B) after activation with MCPyV VLPs. We concluded that PyV VLP-induced inflammasome activation is related to the cathepsin activity, however, other cell activation mechanisms, like K^+ ion efflux, act in concert.

4. DISCUSSION

Macrophages can be activated by various endogenous and exogenous agents, such as bacteria or viral antigens (Mosser and Edwards, 2008). When responding to the activation agents of various structure, phagocytes can provoke the inflammatory reaction. Misfolded protein aggregates are found to be one of the inflammation drivers in the neurodegenerative disorders (Tejera and Heneka, 2016). Aβ represents a DAMP, which is recognized by receptors present in microglia (Venegas and Heneka, 2017). Small oligomers, primary derivatives of Aβ aggregates, induce neuronal loss (Mucke and Selkoe, 2012; Walsh and Selkoe, 2020).

We extended the previously reported research by investigating if soluble Aβ preparations including small oligomers and protofibrils can activate NLRP3 inflammasome in murine brain immune cells – microglia. Both protofibril-forming and lower molecular weight Aβ aggregates induced a significant increase in IL-1β, which is a strong mediator of inflammation, cytokine release and caspase-1 activation which were reduced by NLRP3 inflammasome inhibitor MCC950. Inflammasome activation was confirmed by detection of ASC specks. Therefore, we also showed that Aβ oligomers can act as potent inducers of ASC speck formation. Recently it was reported that ASC specks enhanced inflammatory response in microglia while Aβ and ASC aggregates were formed (Friker *et al.*, 2020). In addition, ASC co-localisation with Aβ in plaques and their boosted cellular activation were detected in AD-relevant mouse model (Venegas *et al.*, 2017). We demonstrated that the inflammasome is activated not only by Aβ fibrils as reported before, but also by low molecular weight oligomers. It is important to mention that size-dependent neurotoxicity of Aβ oligomers is well-documented (Cizas *et al.*, 2010). The neurotoxicity of oligomers used in our work was established in previous studies (Shin *et al.*, 2014; Herzer *et al.*, 2016; Porter *et al.*, 2016).

Our results show that microglia treatment with soluble Aβ oligomers and protofibrils activate all components of the NLRP3 inflammasome inducing an early neuroinflammatory response. However, further mechanism related to Aβ accumulation in the cells may also promote inflammasome activation. Previous studies demonstrated that the uptake of soluble Aβ is facilitated by CD36 and further enhanced by the formation of fibrillary Aβ inside the cell followed by IL-1β secretion in macrophages (Sheedy *et al.*, 2013). Additionally, the activation of the NLRP3 inflammasome induced brain inflammation and reduced clearance of Aβ, while the knockout of NLRP3 in mouse model enhanced Aβ phagocytosis and turned microglia to the anti-inflammatory phenotype (Heneka *et al.*, 2013). Two photon laser scanning

microscopy experiments with APP/PS1 mice of AD model also showed that the knockout of NLRP3 gene enhanced Aβ clearance (Tejera *et al.*, 2019). In addition, it was demonstrated that systemic inflammation induced by LPS facilitated Aβ accumulation, while NLRP3 knockout diminished this effect.

Aβ-induced microglia activation is considered to be associated with phagocytosis and cathepsin release (Lowry and Klegeris, 2018). Inhibition of microglial cathepsin B is supposed to be a neuroprotective treatment in AD as both intracellular and extracellular cathepsins B may enhance the pro-inflammatory effect of microglia. However, it was shown that Aβ oligomers activate microglia via a generation of mitochondrial reactive oxygen species (Parajuli *et al.*, 2013). The use of cathepsin B inhibitor did not change caspase-1 activation and IL-1β release in the microglia cells. Interestingly, Cyt D, the inhibitor of phagocytosis, also did not reduce cellular activation showing the ability of Aβ oligomers to penetrate microglia membrane directly, although in the previous study the phagocytosed fibrillar Aβ induced cathepsin B release from damaged lysosomes (Halle *et al.*, 2008a). In addition, it was demonstrated that inhibition of inflammasome components, caspase-1 and IL-1β, diminished Aβ oligomer-mediated neuronal cell death in the mixed neuronal-glia cell culture (Parajuli *et al.*, 2013). However, the authors did not reveal the influence of buffer used for oligomer preparation on cellular activity. Our experiments showed that lot-to-lot differences of Aβ preparations should be taken into consideration. We could see the influence of buffer solution on cell activation while noxious buffers are used in amyloid preparations. The latter study did not link inflammasome activation and Aβ phagocytosis. However, after microglia treatment with Aβ oligomers we found a small cell population which took up Aβ more intense than other cells and this coincided with a low number of activated microglia cells. Taken all together, Aβ is considered to be an important activating factor of NLRP3 inflammasome, which activation is induced by a variety of signalling pathways.

Most of the studies on cell activation by protein oligomers were carried out using disease-associated protein aggregates such as tau protein, α-synuclein and Aβ. Insoluble structures were deeply investigated but they represent only the last stage of protein aggregation. The effect of primary structures on cell activation could suggest the possible mechanism of pathology onset. However, the mechanisms of cell activation by protein oligomers are not understood in general. It is known that immune cells, like microglia, phagocytose the oligomers and due to the inability to degrade them are stressed. Moreover, aged microglia may become dysfunctional due to the reduction in phagocytosis, autophagic capacity, and impaired reactivity to

antigens (Clayton *et al.*, 2017). Accumulated protein aggregates induce inflammatory response alone or together with other DAMPs (Clark and Vissel, 2015). However, only implicit mechanisms how they activate immune cells are declared.

Research on macrophage activation by polymeric particles and pathogens showed that structural properties is an important factor determining cellular response to the phagocytosed particle (Baranov *et al.*, 2020). Phagocytosis rate of the target is also determined by the target shape and size as silica and latex particles of spherical structure are engulfed faster than ellipsoid particles (Paul *et al.*, 2013). Previous studies demonstrated that nanoparticles composed of carbon and other materials, like cholesterol crystals and monosodium urate crystals, induced lysosomal damage similar to that induced by disease-associated protein aggregates (Nakayama, 2018). The materials effused after lysosomal leakage activate inflammasome and induce inflammation by unknown or complex mechanisms. For studying the mechanisms of macrophage activation, we selected another model of protein oligomers – recombinant viral antigens. We investigated macrophage activation by viral oligomeric proteins of diverse structure (NLPs and VLPs) using human macrophages differentiated from monocytic cell line THP-1.

We did not observe any effect of filamentous NLPs of measles and mumps viruses on the inflammation markers of THP-1 macrophages. In contrast, another study using SARS-CoV-2 N protein demonstrated its capability to promote activation of NLRP3 inflammasome (Pan *et al.*, 2021). In our study spherical VLPs of KIPyV and MCPyV induced the inflammatory response, characterised by IL-6 and TNF-α release, followed by NLRP3 inflammasome activation. We also confirmed PyV-induced inflammasome activation using primary human macrophages. Our results show that viral proteins can activate the inflammasome in human macrophages depending on their structural properties.

In addition, PyV-derived VLPs induced the expression of ASC protein and ASC speck formation proving inflammasome activation. However, ASC specks were not diminished completely after MCC950 treatment. ASC specks can be secreted to the extracellular space and promote IL-1β maturation or act as cell communication molecule (Franklin *et al.*, 2014). Our PyV VLPs could induce ASC speck formation without the assembly of inflammasome complex. The presence of ASC protein in the cells was not reduced by MCC950. Consequently, ASC protein accumulation could occur in an unclear manner, maybe related to the PyV VLPs induced cell signalling.

In summary, VLPs induced NFκ-β signalling pathway and provoked inflammasome assembly following the secretion of inflammatory cytokines

and cell death. We also showed that different size VLPs induce different strength of activation signal. KIPyV-derived VLPs of heterogeneous structure mediated lower cell activation than homogeneous and large MCPyV-derived VLPs. It could be that small VLPs do not activate macrophages or induce only a modest cellular response.

The most important question is what the mechanism of inflammasome activation by PyV-derived VLPs is. As these particles are phagocytosed, they can induce lysosomal damage resulting in processes which activate NLRP3 inflammasome similarly to other nanoparticles that induced cell activation (Shu and Shi, 2018). Inflammasome triggering via cathepsins released after lysosomal damage is one of the possible mechanisms (Rashidi *et al.*, 2020). Treatment of THP-1 macrophages with inhibitors of cathepsins caused a significant decrease in IL-1β levels and cell death induced by PyV VLPs. Inhibitor of cathepsin B CA-074 Me reduced only IL-1β secretion while pan-cathepsin inhibitor K777 down-regulated both IL-1β release and cell death propagated by PyV VLPs. However, neither cathepsin B inhibitor nor pan-cathepsin inhibitor reduced IL-1β levels to control baseline. Previously, cathepsin B was distinguished as a major factor related to a lysosomal damage (Hornung *et al.*, 2008). Later studies showed that other cathepsins also play an important role in activating NLRP3 inflammasome (Campden and Zhang, 2019). In addition, cathepsins might not be a direct factor triggering inflammasome activation. Cell death mediated by certain particles, like monosodium urate crystals, was not reduced by inhibitors of cathepsins (Rashidi *et al.*, 2019). In this case the inhibitor affected only IL-1β release. Other mechanisms stand out beyond or act in concert. For example, cathepsins released after lysosomal rupture induce production of reactive oxygen species, lipid oxidation and other metabolic changes (Wang *et al.*, 2018a). On the other hand, recent study emphasised the role of cathepsin B in NLRP3 activation using cathepsin B-knockout bone marrow-derived macrophages (Chevriaux *et al.*, 2020). In addition, cholesterol content in lysosomes regulate their sensitivity, while depletion of cholesterol promotes cell death (Appelqvist *et al.*, 2012). Thus, particles mediating changes in lysosomal membrane composition may influence cellular activity. Another study demonstrated that the permeabilization of lysosomal membrane induced K+ ion efflux and Ca2+ ion influx following NLRP3 inflammasome activation in murine bone marrow-derived dendritic cell model (Katsnelson *et al.*, 2016). Therefore, we used another inhibitor which blocks K+ ion efflux – one of possible inflammasome activation triggers. K+ ion efflux can be the later process induced by cathepsin-related cell signalling or other processes triggered by VLPs. We found that the inhibitor of K+ ion efflux reduced IL-1β secretion

and cell death induced by PyV VLPs. These results indicate that NLRP3 inflammasome activation by PyV-derived VLPs is partially dependent on cathepsins and other processes stand beyond. Another study also showed that phagocytosed aggregates of prion protein induced NLRP3 inflammasome activation via depletion of intracellular K+ because of lysosome destabilization (Hafner-Bratkovič *et al.*, 2012).

The role of cathepsins in particle-induced cell death is becoming more evident. For example, after treatment of murine macrophages with silica nanoparticles, pan-cathepsin inhibitor K777 reduced IL-1β release, which was confirmed by generation of mature IL-1β form in western-blot. However, cleaved caspase-1 level was reduced partially after K777 treatment. Cathepsin B inhibitor CA-074 Me suppressed IL-1β release in the same way as K777. In addition, K777 significantly reduced other particles, cholesterol crystals, induced formation of cleaved caspase-1 and mature IL-1β. In addition, both K777 and CA-074 Me reduced synthesis of pro-IL-1β and its gene expression induced by LPS pre-treatment (Orlowski *et al.*, 2015). Therefore, mechanism of inflammasome activation by different particles seems to be a complex process as inhibition of cathepsins might reduce IL-1β release due to the generation of a smaller amount of this cytokine precursor. Another research on particle-induced inflammation showed that nanoparticles, like silica, cholesterol crystals and monosodium urate crystals, induced inflammasome-independent cell death. After treatment of murine macrophages, caspase-1, ASC or NLRP3 deficient murine macrophages released IL-1β but at a lower level than wild-type cells (Orlowski *et al.*, 2017). However, cell death was the same in wild-type and NLRP3-deficient cells, except cholesterol crystal case. K777 inhibitor reduced both IL-1β and cell death. After silica treatment, IL-1β release was reduced by both inhibitors Ca074-Me and K777, however, cell death was more selectively reduced by pan-cathepsin inhibitor K777. In addition, silica cytotoxicity was also diminished in macrophages lacking cathepsins B, L, S, or C showing cathepsins role in the inflammation. Therefore, particle-induced inflammatory response seems to be a complex process that might be either dependent or independent on inflammasome components.

To sum up, we demonstrated NLRP3 inflammasome activation by soluble protein oligomers in innate immune cells, macrophages. Our study reveals the importance of structural properties of protein oligomers on cellular response. Macrophage activation by phagocytosed particles is a complex process and different mechanisms can act in concert. This study provides new data on the ability of disease-associated protein oligomers and viral antigens to induce inflammatory response of macrophages.

CONCLUSIONS

1. Aβ oligomers and protofibrils activated NLRP3 inflammasome in the murine microglia showing that primary amyloid derivatives could be a trigger of the neuroinflammatory response.
2. Microglia took up both the Aβ oligomers and protofibrils to the same extent, however, a small population of microglia cells with a significantly higher Aβ content was identified suggesting that some microglia cells have a higher phagocytic activity independently on the Aβ size.
3. A model of filamentous oligomeric proteins – NLPs of measles and mumps viruses did not induce inflammatory response in the murine microglia and human macrophages derived from THP-1 cell line.
4. A model of spherical oligomeric proteins – VLPs of KIPyV and MCPyV triggered inflammatory response followed by NLRP3 inflammasome activation in human THP-1 macrophages and primary human macrophages demonstrating the importance of structural properties of oligomeric proteins on the cell activation.
5. Inhibitors of cathepsins and K+ efflux reduced inflammasome activation induced by VLPs of KIPyV and MCPyV demonstrating that the inflammasome is activated directly by cathepsins released after lysosomal damage or followed by K+ efflux.
6. Different impact of all investigated oligomeric proteins on cellular responses demonstrates that their structural properties are a key factor defining the inflammatory response of macrophages.

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SANTRAUKA

SANTRUMPOS

Aβ – amiloidas beta

AD – Alzhaimerio liga (angl. *Alzheimer's disease*)

ANOVA – dispersinė analizė

ASC – su apoptoze asocijuotas į tašką panašus baltymas, turintis CARD domeną

BSA – jaučio serumo albuminas

DAMPs – organizme susidariusios, su pavojaus signalu susijusios molekulinės struktūros

ELISA – imunofermentinė analizė

FBS – fetalinis veršiuko serumas

GFP – žaliai fluorescuojantis baltymas (angl. *green fluorescent protein*)

HFIP – 1,1,1,3,3,3-heksafluorizopropanolis

IL – interleukinas (pvz., IL-1β, IL-6)

KIPyV – *Karolinska Institutet* poliomos virusas

LDH – laktato dehifrogenazė

LPS – lipopolisacharidas

MCPyV – Merkelio ląstelių poliomos virusas

MeV N – tymų viruso nukleokapsidės baltymas

MuV N – kiaulytės viruso nukleokapsidės baltymas

NLPs – į nukleokapsides panašios dalelės

PAMPs – su patogenais asocijuotos molekulinės struktūros

PBS – fosfatinis buferinis tirpalas

PI – propidžio jodidas

PMA – forbolio 12-miristato 13-acetatas

P/S – penicilinas/streptomicinas

PyV – poliomos virusas

TNF-α – auglio nekrozės faktorius alfa (angl. *tumour necrosis factor*)

VLP – į virusus panašios dalelės

ĮVADAS

Makrofagai yra įgimtojo imuniteto ląstelės, kovojančios su patogenais (Mosser and Edwards, 2008). Jos fagocituoja bakterijas ir kitas pavojingas medžiagas, apoptotines ląsteles bei ląstelių nuolaužas, susidarančias po infekcijos. Makrofagai taip pat atlieka homeostazės palaikymo funkciją audiniuose (Varol *et al.*, 2015). Skirtingai nuo iš monocitų susidariusių makrofagų, kurie atsiranda infekcijų metu, audiniuose esantys makrofagai būna ten visą laiką ir nuolatos tikrina mikroaplinką. Šie makrofagai taip pat sekretuoja signalines molekules audinių ląstelėms bei šalina susidariusias nebereikalingas medžiagas. Infekcijos metu šios ląstelės vienos iš pirmųjų reaguoja į patogenus, praneša apie pavojų kitoms ląstelėms (Zhang *et al.*, 2021a).

Po infekcijos, pašalinus patogeninius veiksnius, sustabdomas uždegiminių molekulių išskyrimas bei vykdoma audinių regeneracija (Muntjewerff *et al.*, 2020). Tačiau stiprios uždegiminės reakcijos metu gali įvykti audinių pažaidos (Parisi *et al.*, 2018). Uždegimą sukeliančiais veiksniais gali būti ne tik patogenai, bet ir įvirios aplinkoje naudojamos medžiagos, pavyzdžiui, anglies, asbesto ar silicio dioksido nanodalelės. Taip pat uždegimą gali sukelti dėl tam tikrų sutrikimų organizme susidarantys junginiai, pavyzdžiui, Alzheimerio ligos (AD) metu susiformuojantys amiloidų beta (Aβ) ir fosforilinto tau baltymo agregatai, cholesterolio ar natrio urato kristalai (Laskin *et al.*, 2011). Makrofagai stengiasi pašalinti šias medžiagas, tačiau jos aktyvina makrofagus ir inicijuoja uždegiminius procesus. Dažniausiai dėl didelio jų kiekio makrofagams sunku jas pašalinti arba dėl to, kad proteazės nėra pajėgios visiškai jas suskaidyti. Besikaupiančios žalingos medžiagos pažeidžia audinius ir sukelia nevaldomą uždegiminį procesą (Watanabe *et al.*, 2019).

Inflamasoma yra viduląstelinis įgimtojo imuniteto komponentas, aktyvinamas įvairiais veiksniais, tiek su patogenais asocijuotomis molekulinėmis struktūromis (PAMPs), pavyzdžiui, LPS, citozoline DNR, poras formuojančiais toksinais; tiek organizme susidariusiomis pavojaus signalo molekulėmis (DAMPs), kurios išskiriamos kaip atsakas į stresą ir audinių pažaidas, pavyzdžiui, cholesterolio kristalai, baltymų agregatai ir ATP (Zheng *et al.*, 2020). Inflamasomą taip pat gali aktyvinti iš aplinkos patenkančios nano- ir mikrodalelės, kaip asbestas. NLRP3 inflamasoma yra geriausiai ištirta ir turi platų spektrą aktyvinančių junginių. Aktyvinimo signalai būna įvairūs, tačiau jie visi sukelia tą patį rezultatą, pavyzdžiui, lizosomų pažaidas, jonų koncentracijų pokyčius ląstelėje ar reaktyvių deguonies formų susidarymą. Šie procesai inicijuoja inflamasomos

susirinkimą. Aktyvinus NLRP3 receptorių, vyksta šių baltymų susirinkimas į multimerinį kompleksą. Prie jo prisijungia ASC baltymai, kurie sudaro platformą kaspazės-1 aktyvinimui. Autoproteolitinio aktyvinimo būdu susidaro aktyvi kaspazė-1, kuri gamina uždegiminius citokinus, pavyzdžiui, IL-1β. Šie citokinai veikia kaip signalinės molekulės inicijuodamos tolimesnį uždegiminį procesą. NLRP3 inflamasomos aktyvinimas dažnai lydimas ląstelės žūtimi – piroptoze. Ši inflamasoma yra siejama su uždegimu virusinių infekcijų ir įvairių patologijų metu, pavyzdžiui, neurodegeneracinių ligų, podagros, vėžio (Wang *et al.,* 2020).

Makrofaguose intensyviai vyksta inflamasomos komponentų raiška ir jos aktyvinimas, kadangi tai yra vienas iš įgimtojo imuniteto apsaugos mechanizmų. NLRP3 inflamasomos aktyvinimas buvo detaliai išanalizuotas makrofaguose naudojant aplinkoje aptinkamas, sintetines polimerines nanodaleles bei PAMP (Kelley *et al.,* 2019). Tačiau mažiau yra žinoma apie inflamasomos aktyvinimo baltymų agregatais mechanizmus. Buvo parodyta, kad netirpios Aβ fibrilės (Halle *et al.,* 2008b), α-sinukleino fibrilės (Codolo *et al.,* 2013) ir tau oligomerai (Ising *et al.,* 2019) aktyvina inflamasomą, tačiau detalūs mechanizmai nėra nustatyti. Taip pat mažai žinoma apie makrofagų aktyvinimą kitais oligomeriniais baltymais.

Įvairios fagocituojamos dalelės gali aktyvinti NLRP3 inflamasomą, pažeidžiant lizosomas (Seoane *et al.,* 2020). Buvo parodyta, kad sintetinės polimerinės dalelės sukelia ląstelių atsaką priklausomai nuo dalelės dydžio, formos ir paviršiaus struktūros (Vaine *et al.,* 2013). Taip pat struktūra nulemia dalelės fagocitozę (Champion and Mitragotri, 2006). Naudojant Aβ buvo parodyta, kad monomerai yra fagocituojami žymiai greičiau nei jų agregatai – protofibrilės (Gouwens *et al.,* 2016). Kitas tyrimas nustatė, kad Aβ fibrilės yra fagocituojamos greičiau nei oligomerai (Pan *et al.,* 2011). Fagocitozė yra nuo receptorių priklausoma endocitozė, tad skirtingos struktūros oligomeriniai baltymai gali įvairiai patekti į ląstelę ir inicijuoti skirtingus signalinius kelius. Pavyzdžiui, α-sinukleino fibrilės jungiasi prie ketvirto tipo komplemento receptoriaus, kuris inicijuoja fibrilių fagocitozę, tačiau monomerų šis receptorius neaptinka (Juul-Madsen *et al.,* 2020). Veiksniai, turintys įtakos fagocitozei, taip pat gali nulemti tolimesnį ląstelių aktyvinimą.

Mūsų tyrime siekėme įvertinti makrofagų aktyvinimą oligomeriniais baltymais uždegimo kontekste. Tyrėme ar ir kaip aktyvinama inflamasoma. Analizavome AD metu susidarančių Aβ agregatų poveikį mikroglijai. Taip pat pasirinkome kitą modelį – rekombinantinius virusinius baltymus, kurie atstovauja egzogeninius antigenus. Virusiniai baltymai plačiai naudojami vakcinacijai (Pollard and Bijker, 2021), tačiau apie jų sąveiką su įgimtojo

imuniteto komponentais žinoma nedaug. Todėl tyrėme, kaip jie aktyvina įgimtojo imuniteto ląsteles – makrofagus.

Tyrimo tikslas: ištirti makrofagų aktyvinimą skirtingos struktūros oligomeriniais baltymais – Aβ agregatais ir virusiniais oligomeriniais baltymais – ir palyginti jų poveikį.

Tyrimo uždaviniai:

1. Įvertinti žmogaus Aβ oligomerų potencialą sukelti uždegiminius procesus makrofaguose, tiriant inflamasomos aktyvinimą.
2. Įvertinti skirtingos struktūros virusinių oligomerinių baltymų potencialą sukelti uždegiminius procesus makrofaguose, tiriant inflamasomos aktyvinimą.
3. Nustatyti inflamasomos aktyvinimo oligomeriniais baltymais mechanizmus.
4. Ištirti sąsajas tarp procesų, susijusių su oligomerinių baltymų fagocitoze ir inflamasomos aktyvinimu.

Mokslinis naujumas

Mūsų tyrimas pateikia naujus mokslinius duomenis apie įgimtojo imuniteto ląstelių sąveiką su organizme susidarančiais baltymų oligomerais – Aβ agregatais, ir su patogenais susijusiais oligomeriniais baltymais –poliomos virusų (PyV) sferinėmis į virusus panašiomis dalelėmis (VLP), sudarytomis iš VP1 baltymo, ir tymų bei kiaulytės virusų filamentinėmis nukleokapsides formuojančiomis dalelėmis (NLP), sudarytomis iš N baltymo. Iki šiol trūksta aiškių įrodymų, ar Aβ tiesiogiai sukelia uždegiminių citokinų susidarymą. Savo tyrime parodėme, kad NLRP3 inflamasoma yra aktyvinama ir mažos molekulinės masės Aβ oligomerais, o ne tik netirpiomis Aβ fibrilėmis, kas buvo nustatyta anksčiau (Halle *et al.,* 2008a). Mūsų rezultatai rodo, kad mikroglijos aktyvinimas šiais pirminiais Aβ agregatais galėtų būti viena iš uždegiminio proceso smegenyse priežasčių prieš susidarant netirpioms amiloidų sankaupoms.

Tirdami virusinius baltymus nustatėme, kad tik PyV VLP sukėlė uždegiminį atsaką žmogaus makrofaguose. NLP neturėjo tokio poveikio. Taigi, parodėme, kad virusiniai baltymai aktyvina NLRP3 inflamasomą priklausomai nuo jų struktūrinių ypatybių. Aktyvinimo mechanizmas yra kompleksinis ir yra susijęs su lizosomų pažaidomis bei K+ jonų išskyrimu iš ląstelių. Anksčiau inflamasomos aktyvinimo mechanizmai buvo detaliai ištirti naudojant polimerines daleles ar kristalus (Rashidi *et al.,* 2020), o mūsų tyrimas parodė fagocituojamų virusinių baltymų sukeliamą poveikį

makrofagams. Tikėtina, kad mūsų nustatyti mechanizmai, kaip oligomeriniai baltymai aktyvina inflamasomą, galėtų būti pritaikomi kitiems baltymams, tarp jų virusiniams antigenams. Dėl to gauti rezultatai galėtų būti svarbūs kuriant vakcinų komponentus.

Mūsų tyrimas parodė heterogeninį makrofagų atsaką į fagocituojamus ir struktūriškai skirtingus oligomerinius baltymus. Tikimės, kad šie rezultatai praplės mechanizmų supratimą – kaip makrofagai aktyvinami įvairiais antigenais ir koks yra inflamasomos vaidmuo makrofagų aktyvinimo procese.

Ginamieji teiginiai

1. Aβ oligomerai ir protofibrilės aktyvino NLRP3 inflamasomą pelės mikroglijos ląstelėse.
2. Tirpūs Aβ agregatai buvo fagocituoti mikroglijos nepriklausomai nuo jų dydžio.
3. Filamentinės struktūros oligomeriniai baltymai – tymų ir kiaulytės virusų nukleokapsides formuojančios struktūros – nesukėlė uždegiminio atsako makrofaguose.
4. Sferinės struktūros oligomeriniai baltymai – poliomos virusų į virusus panašios dalelės – inicijavo uždegiminį atsaką, siejamą su NLRP3 inflamasomos aktyvinimu, žmogaus makrofaguose.
5. Poliomos virusų į virusus panašių dalelių sukeltas inflamasomos aktyvinimo mechanizmas buvo susijęs su katepsinų išskyrimu ir K+ jonų išėjimu iš ląstelės.
6. Struktūrinės oligomerinių baltymų savybės yra vienos iš ląstelinį atsaką nulemiančių veiksnių.

METODAI

Ląstelių kultūros

Pelės pirminė mikroglijos ląstelių kultūra buvo paruošta stažuotės Bonos universitete metu, laikantis Vokietijos gyvūnų gerovės įstatymo. Kultūra buvo ruošiama pagal (Giulian and Baker, 1986) iš 0-3 d. C57BL/6 pelių naujagimių. Smegenys buvo veikiamos tripsinu ir susmulkinamos mechaniškai. Ląstelės buvo sėjamos 75 cm^2 flakonuose (dvejos smegenys per flakoną), naudojant DMEM terpę, papildytą 10 % išaktyvinto fetalinio veršiuko serumo (FBS) bei 1 % penicillino/streptomicino (P/S, 100 U/ml/100 μg/ml). Kitą dieną ląstelės buvo plaunamos fosfatiniu buferiniu tirpalu (PBS) ir pridedama šviežios terpės, papildytos 10 % L929 ląstelių kulūros terpės. Po 8 dienų atkibusios

ląstelės (mikroglija) surenkamos eksperimentams, renkant kas 2-3 dienas iki 3 kartų. Ląstelės užsėtos ½ šviežios terpės ir ½ pirminės kultūros supernatanto. Kitą dieną vykdomas jų aktyvinimas beseruminėje terpėje. Pirmiausia buvo veikiama 3 h 50 ng/mL LPS, o paskui 6 h 2.5 μM Aβ agregatų.

Pelės fibroblastų ląstelių linija L929 buvo kultivuojama DMEM terpėje, papildytoje 1 % P/S ir 10 % FBS. L929 ląstelių supernatantas buvo naudojamas mikroglijos ruošimui. Ląstelės buvo užsėtos esant 1/8 konfluentiškumui ir auginamos 14 dienų. Surinktas supernatantas buvo centrifuguojamas 10 min, 300 × g ir filtruojamas per 0.2 μm porų dydžio filtrą. Paruoštas supernatantas saugomas -20 °C.

Žmogaus monocitų ląstelių linija THP-1, gauta iš dr. Lino Mažučio grupės, buvo kultivuojama RPMI 1640 terpėje, papildytoje 1 % P/S ir 10 % FBS. Eksperimentams buvo naudojamos diferencijuotos ląstelės, toliau vadinamos THP-1 makrofagais. Ląstelių diferenciacija buvo parinkta remiantis šia apžvalga (Chanput *et al.*, 2014). THP-1 monocitai buvo veikiami 48 h 100 ng/ml forbolio 12-miristato 13-acetatu (PMA). Tada PMA buvo pašalintas ir auginama dar 24 h. Ląstelės buvo aktyvinamos beseruminėje RPMI terpėje. Atliekant eksperimentus su virusiniais baltymais buvo naudojama 20 μg/ml koncentracija.

Pirminė žmogaus makrofagų ląstelių kultūra buvo įsigyta iš *Lonza* (#4W-700). Po atšildymo ląstelės augintos RPMI 1640 terpėje, papildytoje 1 % P/S ir 10 % FBS.

NLRP3 inflamasomos slopiklis MCC950 (1 μM) buvo įdėtas prieš poveikį: 20 min Aβ atveju ir 30 min VLP atveju. Nigericinas (10 μM) buvo naudotas kaip teigiama kontrolė.

Ląstelių gyvybingumo įvertinimas

Ląstelių gyvybingumas buvo įvertintas naudojant keletą metodų.

1. Naudojant laktatodehidrogenazės nustatymo rinkinį (LDH, cat#11644793001, Sigma-Aldrich). 50 μL ląstelių supernatanto buvo naudojama šiam testui. Procedūros atliktos pagal gamintojo protokolą.

2. Metabolinis ląstelių aktyvumas buvo įvertintas naudojant XTT metabolinio aktyvumo nustatymo rinkinį (cat#9095, Cell Signaling Technology). XTT metodas buvo atliktas pagal gamintojo protokolą. Inkubacija su reagentais buvo daroma 4 h.

3. Dažant branduolius propidžio jodidu (PI; 1.25 μg/ml) ir Hoechst33342 (1 μg/ml) 30 min. Ląstelės buvo fiksuojamos 4 % paraformaldehidu (PFA). Fluorescencijos signalas nustatytas mikroskopu. Gyvybingumas įvertintas

skaičiuojant PI (žuvusių ląstelių) ir Hoechst (visų ląstelių) santykį ir normalizavus kontrolės atžvilgiu.

IL-1β ir TNF-α sekrecijos nustatymas

Citokinų koncentracija įvertinta imunofermentinės analizės metodu (ELISA).

Citokinų sekrecija pelės mikroglijos ląstelėse buvo nustatyta naudojant šiuos rinkinius: IL-1 beta/IL-1F2 DuoSet ELISA (cat#DY401, R&D Systems), TNF-α DuoSet ELISA kit (cat#DY410, R&D Systems). Surinkti supernatantai buvo saugomi -80 °C.

Citokinų sekrecija THP-1 ląstelėse buvo įvertinta naudojant šiuos rinkinius: Human IL-1 beta Uncoated ELISA Kits, Invitrogen – IL-1β (cat# 88-7261-77), TNF-α (cat#88-7346-86), IL-6 (cat#88-7066) ir IL-10 (cat#88-7106). Surinkti supernatantai buvo saugomi -20 °C.

Citokinų nustatymas atliktas pagal gamintojo protokolą. Citokinų koncentracija apskaičiuota iš kalibracinės kreivės.

Imunocitocheminė analizė

Imunocitocheminei analizei mikroskopu ląstelės buvo auginamos *IbiTreat* plokštelėse. Po poveikio ląstelės buvo 15 min fiksuojamos 4 % PFA. Pašalinus PFA. ląstelės buvo 10 min permeabilizuojamos 0.1 % Triton X-100, toliau blokuojamos 5 % ožkos serumu 30 min. Ląstelės inkubuotos per naktį 4 °C su pirminiais antikūnais blokavimo tirpale. Naudoti šie pirminiai antikūnai: anti-žmogaus/pelės ASC (1:200; cat#AG-25B-0006; AdipoGen), anti-pelės CD68 (1:1000; cat#MCA1957; Bio-Rad), anti-žmogaus CD68 (1:100; #25747-1-AP, Proteintech) ir anti-žmogaus Aβ (3.3 μg/ml; klonas 11E12, (Dalgediene *et al.*, 2013)), anti-PyV VLP ir anti-NLP buvo sukurti mūsų laboratorijoje (hibridomų augimo terpė, skiesta 1:2; #antikūnų klonas – antigenas: #7C11 – MeV N (Zvirbliene *et al.*, 2007); #5E3 – MuV N (Samuel *et al.*, 2002); #5G8 – KIPyV VP1; #11A2 – MCPyV VP1). Po plovimo inkubuota 2 h su atitinkamais antriniais antikūnais. Branduoliai dažyti 30 min 1 μg/mL Hoechst33342. Eksperimentai su Aβ buvo vizualizuoti naudojant Nikon Eclipse Ti mikroskopą, o su VLP ir NLP – EVOS FL Auto fluorescencinį mikroskopą. Nuotraukos apdorotos ir analizuotos *ImageJ* programa.

Aktyvios kaspazės-1 nustatymas FLICA reagentu

Aktyvi kaspazė-1 buvo įvertinta naudojant FLICA rinkinį pagal gamintojo protokolą. Trumpai, FLICA reagentas (FAM-YVAD-FMK – kaspazės-1

slopiklis su fluorescuojančia žyme) buvo įdėtas po ląstelių aktyvinimo tiriamais veiksniais, inkubuota 1 h. Tada ląstelės buvo plaunamos 3 kartus ir dažomos Hoechst 33342 (1 μg/ml), PI (1.25 μg/ml) arba membranos dažu (1:1000; cat#C10046, Invitrogen). Po plovimo ląstelės fiksuotos 4 % PFA. Kaspazės-1 aktyvinimas buvo nustatytas fluorescenciniu mikroskopu. FLICA-kaspazės-1 signalo intensyvumas buvo normalizuotas ląstelių skaičiui.

Tėkmės citometrija

Aβ fagocitozės lygis buvo įvertintas tėkmės citometrijos metodu. Po poveikio mikroglijos ląstelės buvo surinktos, naudojant 0.25 % tripsino-EDTA tirpalą. Ląstelės fiksuotos 4 % PFA (10 min) ir permeabilizuotos 0.03 % Triton X-100 tirpalu (5 min), blokuoti paviršiaus receptoriai FcγRIII (CD16) ir FcγRII (CD32) anti-pelės CD16/32 reagentu (cat#553141, BD). Tada ląstelės buvo dažomos pirminiais antikūnais anti-Aβ (klonas 11E12) per naktį, 4 °C. Antriniai antikūnai (1:600; cat#A11017, Invitrogen) buvo inkubuojami 1 h kartu su anti-CD11b-APC (1:100; cat#101212, Biolegend), 4 °C. Po plovimo ląstelės buvo suspenduotos dažymo buferiniame tirpale (2 % FBS PBS'e). Mėginiai matuoti *BD FACSCanto II* tėkmės citometru ir analizuoti *FlowJo* programa.

Imunoblotas

Aktyvios kaspazės-1 20 kDa fragmentas, vadinamas p20, buvo nustatytas imunoblotu. Surinkti ląstelių supernatantai centrifuguoti 600 × g 10 min ir 10x sukoncentruoti, naudojant 3 kDa dydžio (mikroglijos, cat#UFC500396, Amicon) arba 10 kDa dydžio (THP-1 ląstelių, cat#UFC501096, Amicon) ultrafiltravimo mėgintuvėlius.

Pelės mikroglijos ląstelių p20 nustatytas naudojant *Jess system* imunobloto prietaisą (ProteinSimple, Bio-Techne, JAV). Procedūros atliktos naudojant gamintojo reagentus ir protokolą (*12-230 kDa Jess/Wes Separation Module*) chemoliuminescenciniu metodu. Tyrimui buvo naudojama 20 μg/ml antikūnų prieš kaspazę-1 (cat#AG-20B-0042, AdipoGen).

THP-1 ląstelių p20 fragmentas nustatytas įprastu imunoblotu. Mėginiai buvo redukuojami ir atskiriami 4-12% poliakrilamidiniame gelyje. Pernešimui naudota 0.2 μm nitroceliuliozinė membrana. Membrana blokuota 3 % BSA tirpalu PBS 1 h kambario temperatūroje, tada inkubuota su pirminiais antikūnais (1:1000; cat#AG-20B-0048-C100, Adipogene) per naktį, 4 °C. Su antriniais antikūnais (1:5000; cat#1706516, Bio-Rad)

inkubuota 1 h kambario temperatūroje. Membranos ryškinimui naudotas chemoliuminescencinis substratas (cat#34094, Thermofisher Scientific). Vizualizuota *ChemiDoc Imaging System* prietaisu (Bio-Rad).

$A\beta_{1-42}$ paruošimas

Aβ oligomerai ir protofibrilės buvo paruošti pagal aksčiau sukurtą protokolą (Stine *et al.*, 2003). Aβ buvo ištirpintas HFIP iki 221 μM konentraijos. 100 μl ruošiniai buvo inkubuoti kambario teperatūroje. HFIP išgarintas, naudojant vakuuminę centrifugą 800 × g, 10 min. Paruošti peptidų mėginiai buvo saugomi -80 °C. Šie mėginiai buvo tirpinami DMSO iki 2.5 mM koncentracijos ir sonikuoti 10 min. Aβ arba DMSO kontrolė buvo įdėta į fenolio raudonojo neturinčią DMEM terpę (galutinė koncentracija 100 μM) ir inkubuota 24 h 4 °C (oligomerai) arba 37 °C (protofibrilės). Aβ struktūra buvo charakterizuota atominės jėgos mikroskopu.

PyV VP1 VLP ir tymų bei kiaulytės N baltymai

Rekombinantiniai baltymai buvo gauti naudojant *Saccharomyces cerevisiae* raiškos sistemą. PyV VP1 VLP buvo ruošiamos pagal ankstesnę metodiką VU GMC Eukariotų ir genų inžinerijos skyriuje (Norkiene *et al.*, 2015a). Kiaulytės (cat#12MuNP-ASc-Gly-C) ir tymų (cat#12MeN-BSc-Gly-C) N baltymai buvo įsigyti iš UAB Baltymas (Lietuva). Baltymų monomerų dydžiai: MCPyV VP1 MW = 46.56 kDa; KIPyV VP1 MW = 41.59 kDa; MeV N MW = 58 kDa; MuV N MW = 66 kDa.

ASC dalelių nustatymas naudojant THP1-ASC-GFP ląsteles

THP1-ASC-GFP ląstelės vykdo ASC baltymo, konjuguoto su žaliai fluorescuojančiu baltymu (GFP), raišką. Aktyvinus NF-kB kelią, ląstelėse matomas žalias fluorescencijos signalas, rodantis ASC-GFP sintezę. Susirinkus inflamasomai, matomos ASC-GFP polimerų suformuotos dalelės.

THP1-ASC-GFP buvo auginama taip pat kaip THP-1 ląstelių linija, tik naudojant papildomus antibiotikus – 100 μg/ml zeociną ir 100 μg/ml normociną. Makrofagai buvo veikiami virusiniais baltymais (20 μg/ml) 24 h beseruminėje RPMI 1640 terpėje. Po poveikio buvo dažomi branduoliai Hoechst33342, ASC dalelės vizualizuojamos fluorescenciniu mikroskopu. Duomenys analizuoti *ImageJ* programa. ASC dalelių kiekis normalizuotas ląstelių skaičiui.

Statistinė analizė

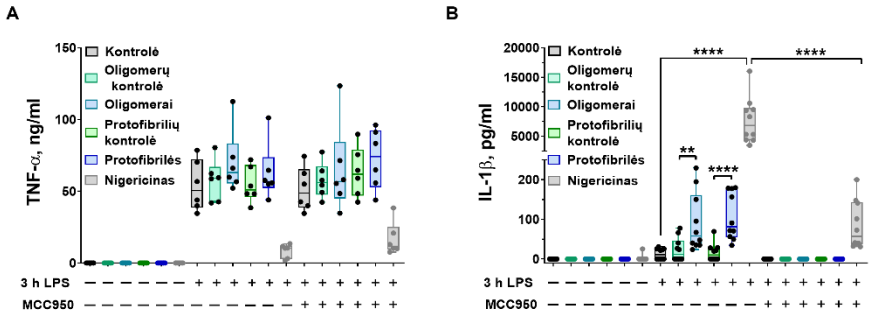
Duomenys iš bent 3 nepriklausomų eksperimentų ar techninių pakartojimų buvo analizuojami *9.3.1 GraphPad Prism* programa. Duomenys pavaizduoti stačiakampėmis diagramomis arba stulpelinėmis diagramomis su individualiais taškais. Buvo tikrinama, ar duomenys atitinka Gauso skirstinį. Jei taip, ANOVA palyginimas buvo atliekamas naudojant *Bonfferoni* arba *Tukey* testus. Jei duomenys nebuvo išsibarstę pagal normalųjį skirstinį, buvo atliekamas palyginimas *Kruskal-Wallis* metodu kartu su *Dunn's post hoc* testu. Jei p vertės buvo mažesnėms už 0.05, buvo laikoma statistiškai reikšmingu skirtumu ir nurodoma taip: *$p < 0.05$, **$p<0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Siekėme nustatyti, ar oligomeriniai baltymai sukelia uždegiminį atsaką makrofagų ląstelėse, bei ištirti, ar šie baltymai aktyvina inflamasomą. Tyrėme skirtingos struktūros ir kilmės oligomerus: Aβ oligomerus kaip endogeninius antigenus ir virusinius baltymus kaip organizmui svetimus (egzogeninius) antigenus.

Mikroglijos aktyvinimo Aβ oligomerais tyrimas

Siekėme ištirti, ar tirpūs Aβ agregatai – maži oligomerai ir protofibrilės – aktyvina NLRP3 inflamasomą makrofaguose. Šių agregatų neurotoksiškumas buvo parodytas kituose tyrimuose (Shin *et al.*, 2014; Herzer *et al.*, 2016; Porter *et al.*, 2016). Tirpių Aβ agregatai susidaro vieni iš pirmųjų AD metu, todėl galėtų būti uždegimo šaltinis. Naudojome skirtingos agregacijos lygio Aβ oligomerus, kurie galėtų sukelti nevienodą poveikį. Tirdami NLRP3 inflamasomos aktyvinimą naudojome specifinį jos slopiklį MCC950. Nigericinas buvo naudotas kaip teigiama inflamasomos aktyvinimo kontrolė. Kadangi Aβ dalyvauja neurodegeneracinių ligų patogenezėje, tyrimų modelis buvo pelės pirminė mikroglijos ląstelių kultūra.

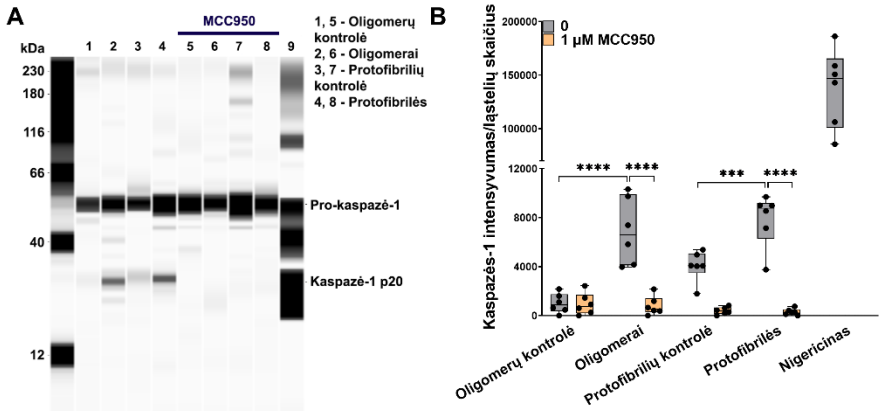
Aktyvinus mikrogliją Aβ oligomerais ir protofibrilėmis, nenustatėme uždegiminio atsako (Pav. 1). Dėl to mikroglija buvo pirmiausia aktyvinta LPS, siekiant indukuoti inflamasomos komponentų raišką. Tada ląstelės buvo veiktos Aβ agregatais 6 h. LPS sukėlė TNF-α sekreciją, tačiau Aβ pridėjimas neturėjo įtakos šio citokino sekrecijai (Pav. 1A). Taip pat nustatėme IL-1β sekreciją po poveikio tiek Aβ oligomerais, tiek protofibrilėmis (Pav. 1B). Specifinis NLRP3 slopiklis MCC950 visiškai nuslopino IL-1β sekreciją – tai rodo inflamasomos aktyvinimą.



1 pav. Aβ oligomerai ir protofibrilės sukėlė IL-1β sekreciją mikroglijoje. Mikroglija buvo aktyvinama LPS prieš Aβ poveikį. Citokinų sekrecija buvo matuojama supernatantuose ELISA metodu: (A) IL-1β, n = 10 ir (B) TNF-α,

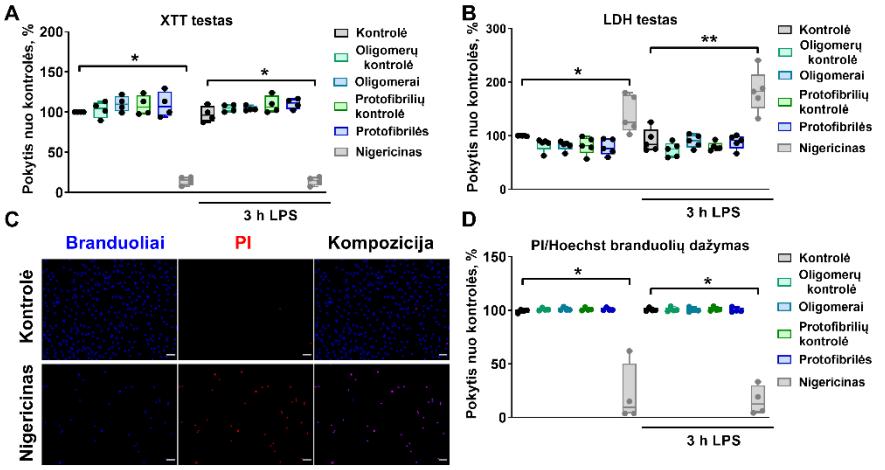
n = 6. **p < 0.01, ****p < 0.0001 – pagal ANOVA, atliekant *Bonferroni's* testą.

Aβ oligomerai ir protofibrilės taip pat sukėlė proteolitinį kaspazės-1 aktyvinimą, kurį parodė p20 fragmento susidarymas (Fig. 2). NLRP3 inflamasomos slopiklis MCC950 sumažino p20 kiekį, o tai patvirtina inflamasomos aktyvinimą Aβ.



2 pav. Aβ sukėlė aktyvios kaspazės-1 fragmento p20 susidarymą mikroglijoje. Mikroglija buvo aktyvinama LPS prieš Aβ poveikį. Kaspazės-1 p20 fragmentas nustatytas imunobloto metodu. (A) Reprezentacinis imunoblotas. (B) Kaspazės-1 p20 skaitinis įvertinimas, n = 6. ***p < 0.001, ****p < 0.0001, pagal ANOVA, atliekant *Bonferroni's* testą.

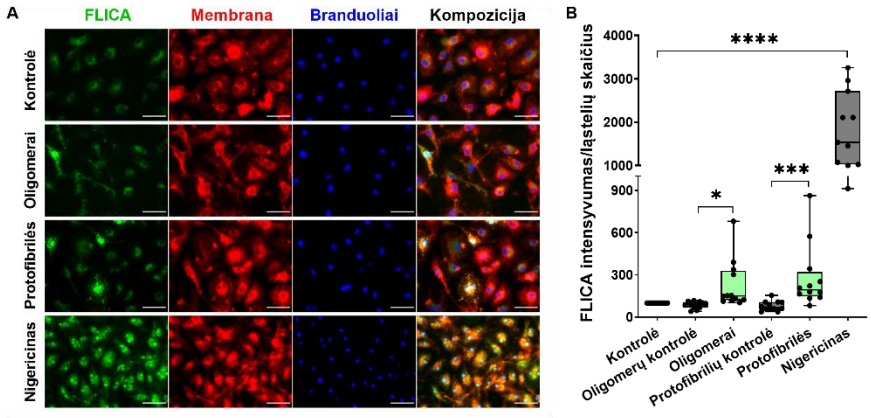
Inflamasomos aktyvinimas dažnai pasibaigia ląstelių žūtimi – piroptoze. Tačiau nei metabolinio aktyvumo nustatymo testas XTT, nei LDH išskyrimo testas, nei branduolių dažymas PI neparodė statistiškai reikšmingų gyvybingumo pokyčių (Pav. 3ABCD). Todėl daroma išvada, kad Aβ agregatai nesukelia mikroglijos žūties arba pokytis per mažas, kad būtų aptinkamas. Palyginus ląstelių gyvybingumo pokyčius po poveikio klasikiniu inflamasomos aktyvikliu nigericinu (Pav. 3), matome, kad Aβ sukėlė nedidelį ląstelių atsaką, taip pat lyginant IL-1β sekreciją ir kaspazės-1 aktyvinimą (Pav. 1 ir 2).



3 pav. Aβ neturėjo poveikio mikroglijos gyvybingumui. Mikroglija buvo aktyvinama LPS prieš Aβ poveikį. Citotoksiškumo įvertinimui naudotas keletas testų: (A) Metabolinio aktyvumo XTT metodas (n = 4), (B) LDH testas (n = 5), ir (C, D) Branduolių dažymas PI ir Hoechst (n = 4). (c) Reprezentacinės nuotraukos, PI (žuvusiais ląstelės), Hoechst (visos ląstelės). Skalė – 40 μm. (D) Branduolių dažymo skaitinis įvertinimas. *p < 0.05, **p < 0.01, pagal *Kruskal–Wallis* testą kartu su *Dunn's post hoc* testu.

Siekiant nustatyti aktyvintų mikroglijos ląstelių kiekį, atlikome aktyvios kaspazės nustatymą vienos ląstelės lygyje, naudojant FLICA reagentą. Šis reagentas turi FAM-YVAD-FMK – kaspazės-1 fluorescuojantį slopiklį, kuris jungiasi tik prie aktyvios kaspazės-1. Palyginus su nigericinu, nustatėme nedidelį aktyvintų ląstelių kiekį (Pav. 4). Taigi, rezultatai rodo, kad Aβ agregatais indukuoja inflamasomą tik tam tikrose ląstelėse. Taip pat nebuvo nustatyta skirtumų tarp Aβ oligomerų ir protofibrilių. Galime daryti išvadą, kad tiek tirpūs Aβ agregatai aktyvina inflamasomą, tiek anksčiau ištirtos netirpios Aβ fibrilės (Halle *et al.*, 2008a).

Taigi, nustatėme, kad tirti Aβ agregatai aktyvino mikrogliją, sukeldami uždegiminio citokino IL-1β sekreciją bei kaspazės-1 aktyvinimą. Įdomu, kad buvo aktyvinta tik maža dalis ląstelių. Savo tyrimu parodėme, kad inflamasoma yra aktyvinama ne tik netirpiomis Aβ fibrilėmis, kas jau buvo parodyta anksčiau (Halle *et al.*, 2008b), bet ir mažos molekulinės masės oligomerais. Amiloidų dydis lemia neurotoksiškumą (Cizas *et al.*, 2010), tačiau inflamasomos aktyvinimui vyksta nepriklausomai nuo jų dydžio, galbūt skirtingu mechanizmu.

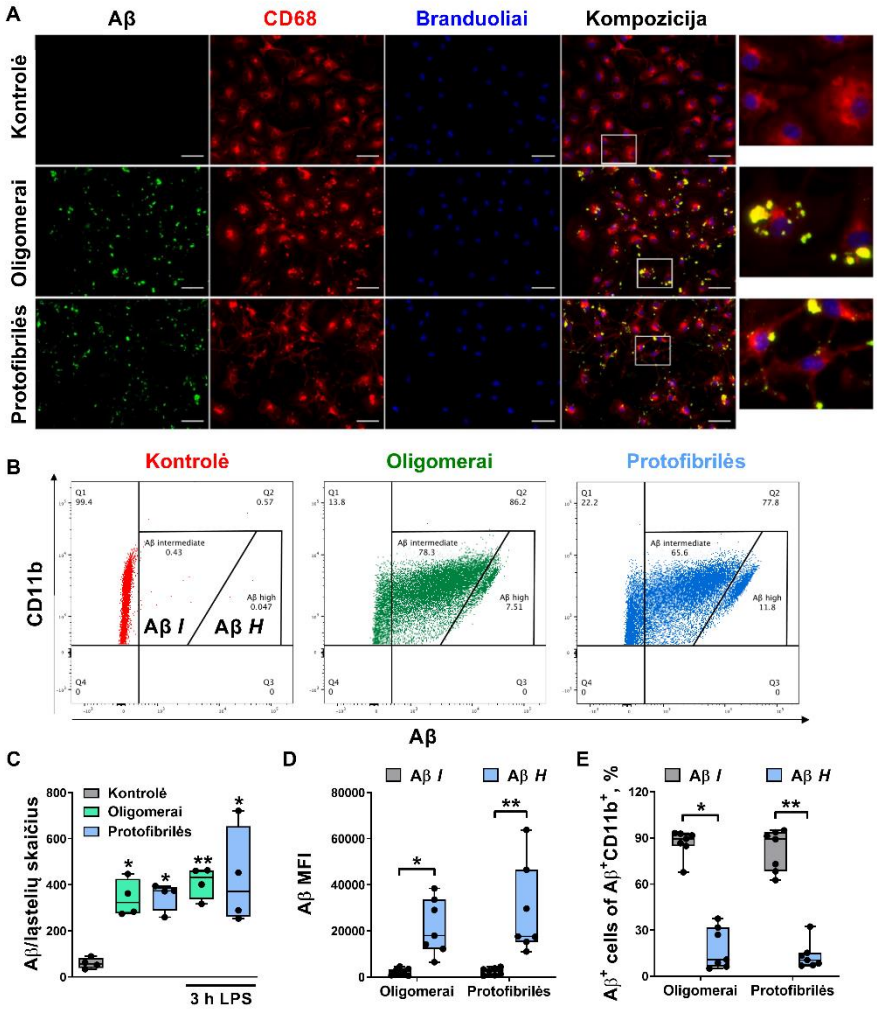


4 pav. Aβ sukėlė kaspazės-1 aktyvinimą mikroglijoje. Mikroglija buvo aktyvinama LPS prieš Aβ poveikį. Ląstelės buvo dažomos FLICA reagentu (aktyvi kaspazė-1), membranos dažu; branduoliai – Hoechst. (A) Reprezentacinės nuotraukos. Skalė – 40 μm. (B) Kaspazės-1 skaitinis įvertinimas (n = 12). *p < 0.05, ***p < 0.001, ****p < 0.0001, pagal *Kruskal–Wallis* testą kartu su *Dunn's post hoc* testu.

Nustatėme, kad NLRP3 inflamasoma yra aktyvinama pirminiais amiloidų dariniais. Tai rodo, kad inflamasomos aktyvinimas šiais oligomerais galėtų būti neuroužd egimo pradžia. Žinoma, kad NLRP3 geno pašalinimas AD pelių modelyje sumažina uždegimą ir sustiprina Aβ fagocitozę mikroglijos ląstelėse (Heneka *et al.*, 2013; Tejera *et al.*, 2019). Aβ sukeltas mikroglijos aktyvinimas siejamas su fagocitozės procesu ir katepsinų poveikiu (Halle *et al.*, 2008a; Lowry and Klegeris, 2018). Pažeidus šias lizosomas, išsilieja katepsinai, kurie gali inicijuoti kitus procesus, kaip reaktyvių deguonies formų susidarymas, o šie savo ruožtu sužadina inflamasomą.

Kadangi inflamasomos aktyvinimo Aβ mechanizmas yra siejamas su fagocitoze, tyrėme Aβ oligomerų ir protofibrilių fagocitozę imunocitochemijos metodu. CD68 buvo naudojamas kaip makrofagų ir lizosomų žymuo. Siekiant įvertinti fagocitozės lygį, analizavome Aβ lokalizaciją su CD68. Nustatėme, kad Aβ oligomerai ir protofibrilės buvo endocituoti (Pav. 5AC), tačiau nematėme skirtumų tarp jų fagocitozės lygio. Toliau atlikome Aβ fagocitozės analizę tėkmės citometru. Nustatėme vienodą abiejų Aβ formų fagocitozę (Pav. 5B), tačiau taip pat atsiskyrė maža mikroglijos ląstelių populiacija, kuri fagocitavo daugiau Aβ (Pav. 5DE). Manome, kad ši populiacija galėtų reprezentuoti ląsteles, pasižyminčias didesniu fagocitiniu aktyvumu. Taip pat ši populiacija sutampa su nedideliu Aβ aktyvintų ląstelių kiekiu (kaspazės-1 aktyvinimas, IL-1β sekrecija),

lyginant su stipriu aktyvikliu nigericinu. Tai rodo, kad inflamasomos aktyvinimas Aβ agregatais yra susijęs su jų fagocitozės intensyvumu, tačiau galimi įvairūs mikroglijos aktyvinimo keliai.



5 pav. Mikroglijos vydoma Aβ oligomerų ir protofibrilių fagoitozė. Mikroglija buvo aktyvinama LPS prieš Aβ poveikį. Ląstelės buvo dažomos anti-Aβ, anti-CD68; branduoliai – Hoechst. (A) Reprezentacinės imunocitocheminės analizės nuotraukos. Skalė – 40 μm. (C) Aβ fagocitozės apskaičiavimas pagal imunocitocheminę analizę – Aβ lokalizacija su CD68, n = 4. Tėkmės citometrijos analizei ląstelės buvo dažoma anti-Aβ ir anti-CD11b. Buvo analizuojamos $CD11b^{+}Aβ^{+}$ ląstelės. (B) Reprezentacinis tėkmės citometrijos paveikslas. (D) ir (E) Aβ fagocitozės tėkmės citometrijos metodu skaitinis įvertinimas. $CD11b^{+}Aβ^{+}$ populiacija, fagocitavusi daugiau

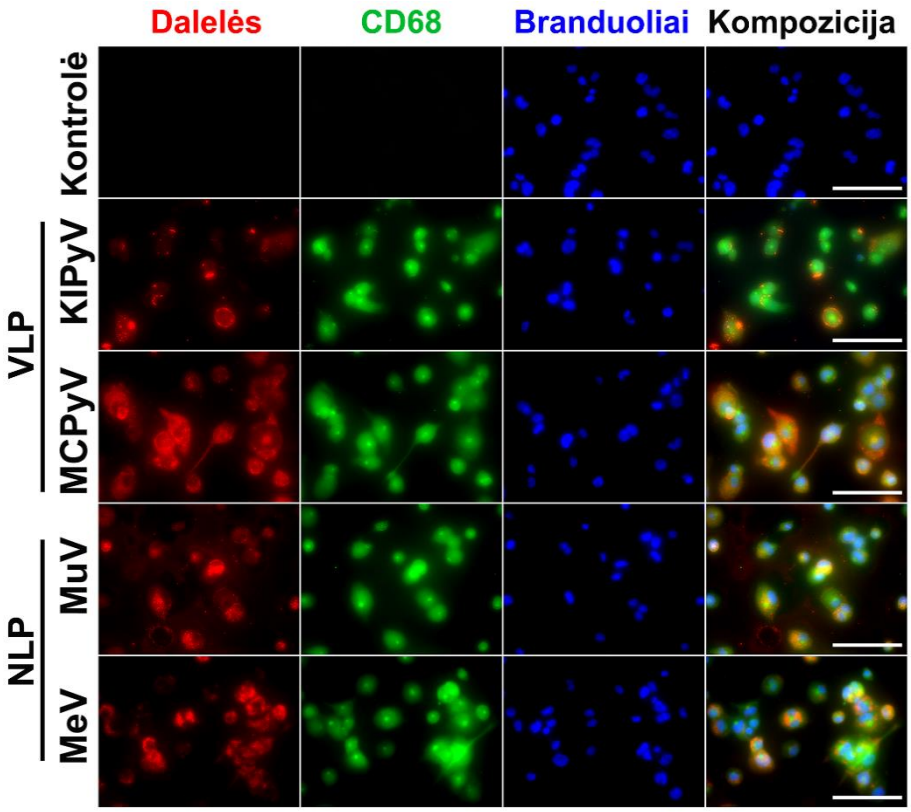
Aβ, buvo žymima Aβ *H*. Kitos ląstelės – Aβ *I*. (D) $CD11b^{+}A\beta^{+}$ ląstelių kiekis ir (E) MFI – fluorescencijos intensyvumo mediana skirtingose populiacijose, n = 7. *p < 0.05, **p < 0.01, mikroskopijai pagal *Kruskal–Wallis* testą kartu su *Dunn's post hoc* testu; tėkmės citometrijai pagal ANOVA, atliekant *Bonferroni's* testą.

Makrofagų aktyvinimo virusiniais oligomeriniais baltymais tyrimas

Siekdami nustatyti makrofagų aktyvinimo mechanizmus, pasirinkome skirtingos struktūros virusinius oligomerinius baltymus. Tyrėme ar jie sukelia uždegiminį atsaką ir aktyvina inflamasomą. Pasirinkome žmogaus ląstelių modelį – THP-1 makrofagus.

Tyrėme skirtingos struktūros virusinius baltymus, siekiant įvertinti struktūrinių ypatybių svarbą ląstelių aktyvinimui. Vienas iš modelių buvo rekombinantiniai tymų (MeV) ir kiaulytės virusų (MuV) filamentines/lazdelės formos struktūras formuojantys N baltymų oligomerai. Jie formuoja apie 20 nm skersmens NLP (Samuel *et al.*, 2002; Slibinskas *et al.*, 2004). Kitas modelis – PyV VP1 baltymų oligomerai. VP1 baltymas susirenka į maždaug 360 monomerų sferines VLP, kurios imituoja viruso kapsidę. Tyrėme rekombinantines žmogaus KI ir MC PyV VLPs. KIPyV VLPs formuoja heterogeniškas, 20-60 nm skersmens VLPs, MCPyV – homogenines, 45-50 nm skersmens VLP.

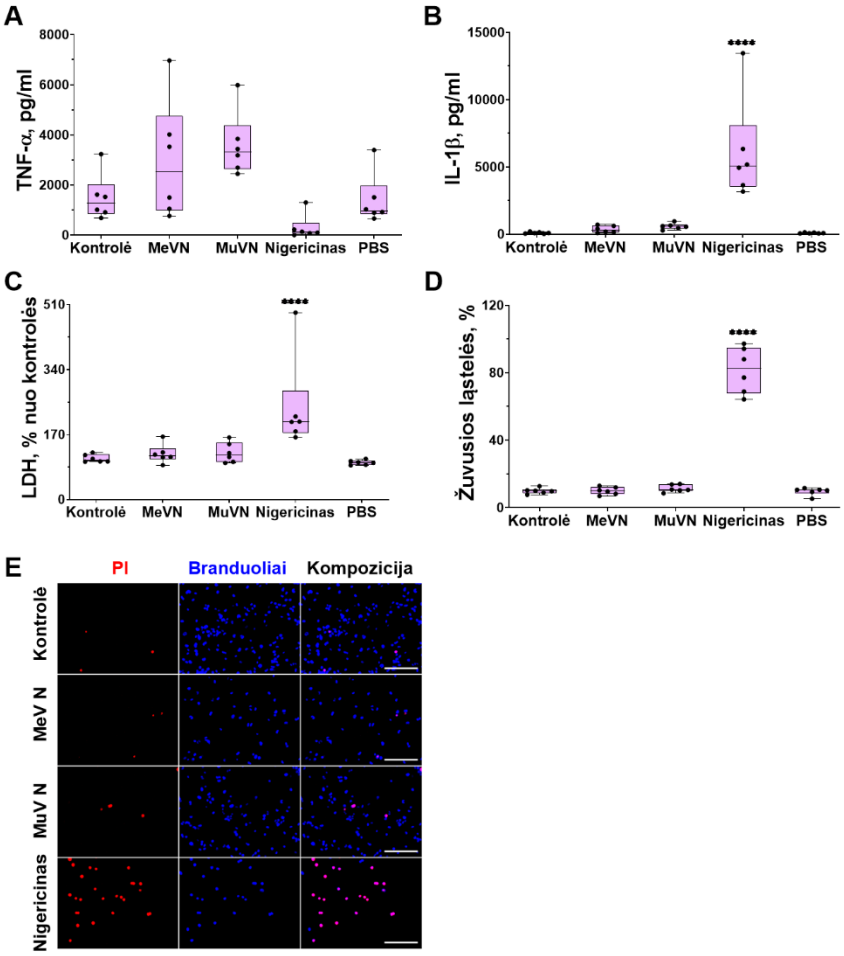
Pirmasis aktyvinimo etapas yra ląstelių sąveika su antigenu. Todėl pradėjome nuo imunocitocheminės analizės, siekiant nustatyti, ar virusiniai oligomeriniai baltymai patenka į THP-1 makrofagus. Pagal VLP ir NLP signalo lokalizaciją su CD68 nustatėme šių dalelių fagocitozę (Pav. 6). Taigi, visi tiriami baltymai yra aptinkami makrofagų.



6 pav. THP-1 makrofagų vykdoma tymų ir kiaulytės virusų NLP ir PyV VLP fagocitozė. Makrofagai buvo veikiami 24 h virusiniais baltymais. Ląstelės buvo dažytos anti-NLP, anti-VLP ir anti-CD68; branduoliai – Hoechst33342. Skalė – 100 μm. Pateiktos reprezentacinės nuotraukos.

Makrofagų aktyvinimo virusiniais baltymais tyrimą pradėjome nuo tymų ir kiaulytės virusų NLP. Po THP-1 makrofagų veikimo rekombinantiniais baltymais, vertinome uždegiminių citokinų IL-1β ir TNF-α sekreciją bei ląstelių gyvybingumą. Pagal šiuos parametrus nenustatėme ląstelių aktyvinimo NLP (Pav. 7). Taigi, tirtieji filamentinės struktūros virusiniai baltymai nesukelia uždegiminio atsako makrofaguose.

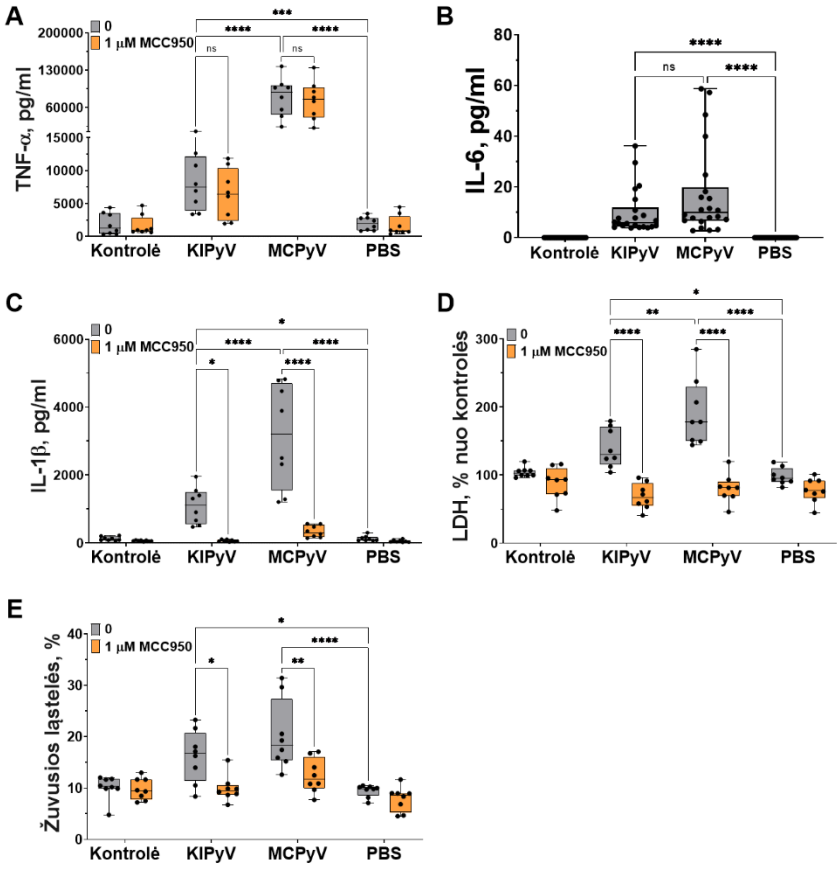
Mūsų tirti filamentinės struktūros tymų ir kiaulytės virusų NLP nesukėlė uždegiminio atsako THP-1 makrofaguose. Tačiau kitas tyrimas, kuriame buvo naudoti SARS-CoV-2 N baltymai, parodė, kad šis baltymas gali aktyvinti NLRP3 inflamasomą (Pan *et al.*, 2021). Šį skirtumą galėjo lemti N baltymų struktūriniai skirtumai.



7 pav. Tymų ir kiaulytės virusų NLP nesukėlė uždegiminio atsako THP-1 makrofaguose. THP-1 makrofagai buvo veikiami 24 h NLP. Citokinų sekrecija buvo matuojama supernatantuose ELISA metodu: (A) TNF-α ir (B) IL-1β, n = 6. Citotoksiškumas įvertintas (C) LDH testu (n = 5), ir (D, E) Branduolių dažymu PI (žuvusios ląstelės) ir Hoechst (visos ląstelės). (E) Reprezentacinės dažymo nuotraukos. Skalė – 200 μm. (D) Branduolių dažymo skaitinis įvertinimas. n = 6, ****p < 0.0001 – pagal ANOVA, atliekant *Tukey's* testą, skirtumai parodyti kontrolės atžvilgiu.

Toliau tyrėme makrofagų aktyvinimą rekombinantinėmis PyV VLP, formuojančiomis sferines struktūras. Tiek KIPyV, tiek MCPyV VLP sukėlė uždegiminių citokinų IL-6 ir TNF-α sekreciją (Pav. 8AB). Didesnės MCPyV VLP skatino didesnę TNF-α sekreciją. Taip pat tyrėme priešuždegiminio

citokino IL-10 išskyrimą, tačiau jo neaptikome. Taigi, priešingai nei NLP, PyV VLP sukėlė uždegiminį atsaką THP-1 makrofaguose.

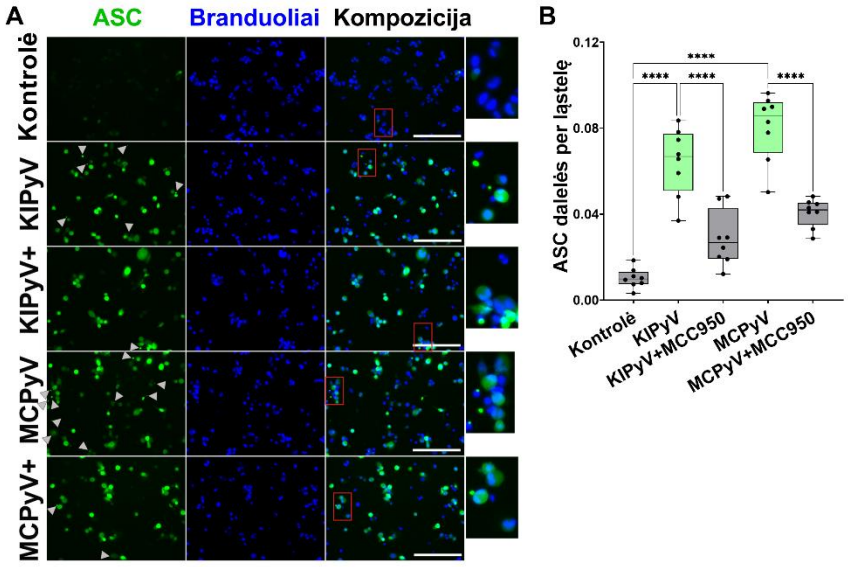


8 pav. PyV VLP sukėlė uždegiminių citokinų sekreciją bei aktyvino NLRP3 inflamasomą THP-1 makrofaguose. THP-1 makrofagai buvo veikiami 24 h PyV VLP. Citokinų sekrecija buvo matuojama ląstelių supernatantuose ELISA metodu: (A) TNF-α, (B) IL-6 (n = 22) ir (C) IL-1β. Citotoksiškumas įvertintas (D) LDH testu, ir (E) Branduolių dažymu PI (žuvusios ląstelės) ir Hoechst (visos ląstelės). n = 8, *p < 0,05, **p < 0,01, ***p < 0,001, ****p < 0,0001 – pagal ANOVA, atliekant *Tukey's* testą.

Siekėme nustatyti, ar PyV VLP aktyvina NLRP3 inflamasomą THP-1 makrofagusoe. KIPyV ir MCPyV VLP sukėlė IL-β sekreciją (Pav. 8C) bei ląstelių žūtį (Fig. 8DE). Iš branduolių dažymo PI matome, kad tik apie 10 % ląstelių žuvo, tad tik dalis ląstelių buvo aktyvinta. Specifinis NLRP3 inflamasomos slopiklis MCC950 visiškai nuslopino šį atsaką – tai rodo, kad PyV VLP aktyvina inflamasomą. Taip pat MCPyV VLP skatino didesnį makrofagų aktyvinimą nei KIPyV VLP.

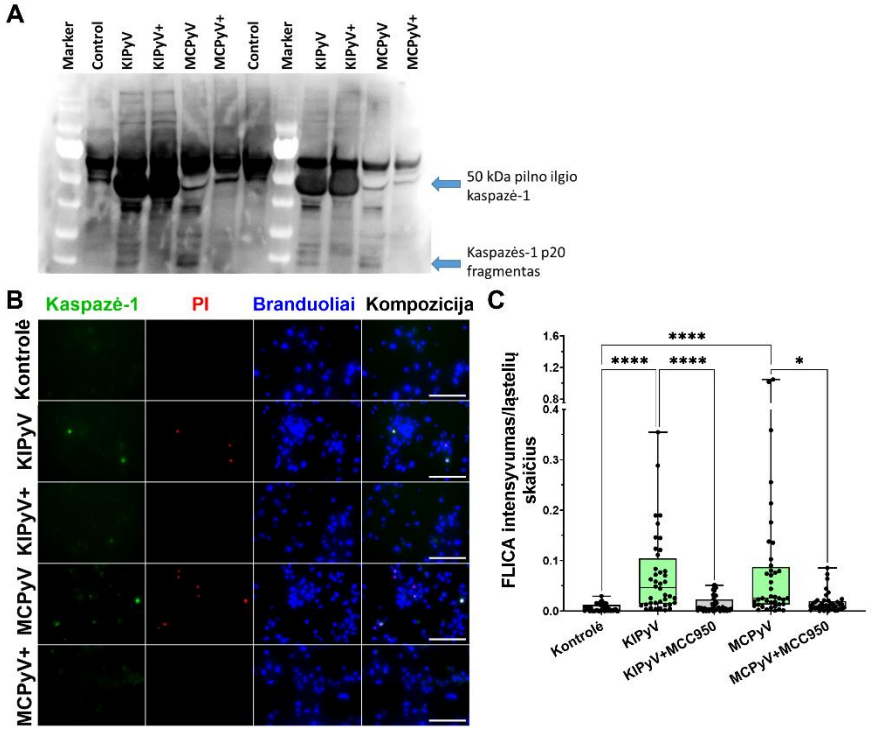
NLRP3 inflamasoma sudaryta iš ASC baltymų, kurie veikia kaip platforma inflamasomos susirinkimui. Žinoma, kad ASC baltymų agregatai gali susidaryti šalia inflamasomos susirinkimo ir būti sekretuojami už ląstelės ribų bei skatinti uždegiminę reakciją (Franklin *et al.*, 2014). Siekdami įvertinti ASC baltymo sankaupų (dalelių) ir inflamasomos susidarymą, naudojome THP-1-ASC-GFP ląsteles, sintetinančias žaliai fluorescuojantį ASC baltymą. ASC baltymo sintezė vyksta po NFκ-β aktyvinimo (Hoss *et al.*, 2017). THP-1-ASC-GFP ląstelėse nustatytas fluorescencinis signalas po poveikio PyV VLP (Pav. 10A) – tai rodo, kad šie oligomeriniai baltymai aktyvina NFκ-β signalinį kelią ir skatina ASC baltymo sintezę.

Tirtos PyV VLP sukėlė ASC dalelių susidarymą (Pav. 9) THP-1-ASC-GFP makrofaguose. Slopiklis MCC950 reikšmingai sumažino šių dalelių susidarymą, o tai patvirtina inflamasomos aktyvinimą (Pav. 9B). Tačiau MCC950 visiškai nenuslopino ASC dalelių formavimosi. MCC950 slopiklis pačios ASC baltymo sintezės neslopino. PyV VLP galėjo skatinti ASC baltymo sankaupų susidarymą. Taip pat gali būti, kad VLP stipriai aktyvina ląsteles ir kitu mechanizmu skatina ASC dalelių formavimąsi.



9 pav. PyV VLP sukėlė ASC dalelių susidarymą THP-1 makrofaguose. THP-1-ASC-GFP makrofagai buvo veikiami 24 h PyV VLP. ASC dalelių formavimasis stebėtas mikroskopu. (A) Reprezentacinės nuotraukos. ASC dalelės pažymėtos rodyklėmis. Skalė – 200 μm. (B) ASC dalelių skaitinis įvertinimas. n = 8, *p < 0.05, ****p < 0.0001 – pagal ANOVA, atliekant *Tukey's* testą.

Inflamasomos aktyvinimo metu aktyvi kaspazė-1 katalizuoja IL-1β susidarymą iš jo pirminės formos. Dėl to tyrėme aktyvios kaspazės fragmento p20 susidarymą ląstelių supernatante. PyV VLP sukėlė kaspazės-1 aktyvinimą, o MCC950 jį nuslopino (Pav. 10A). Taip pat siekėme įvertinti aktyvios kaspazės-1 susidarymą ląstelėse, panaudojant FLICA reagentą. Jis turi FAM-YVAD-FMK – kaspazės-1 slopiklį, kuris prisijungia tik prie aktyvintos kaspazės-1. Šiuo metodu taip pat parodėme aktyvios kaspazės-1 susidarymą (Pav. 10B). Slopiklis MCC950 reikšmingai sumažino šį poveikį (Pav. 10C). Šiuo tyrimu patvirtinome, kad PyV VLPs aktyvina NLRP3 inflamasomą makrofaguose. Taip pat FLICA metodu nustatėme, kad tik maža ląstelių dalis yra aktyvinama (Pav. 10B). PI dažymas parodė, kad FLICA reagentu nusidažiusios ląstelės buvo žuvusios – tai rodo piroptozę.

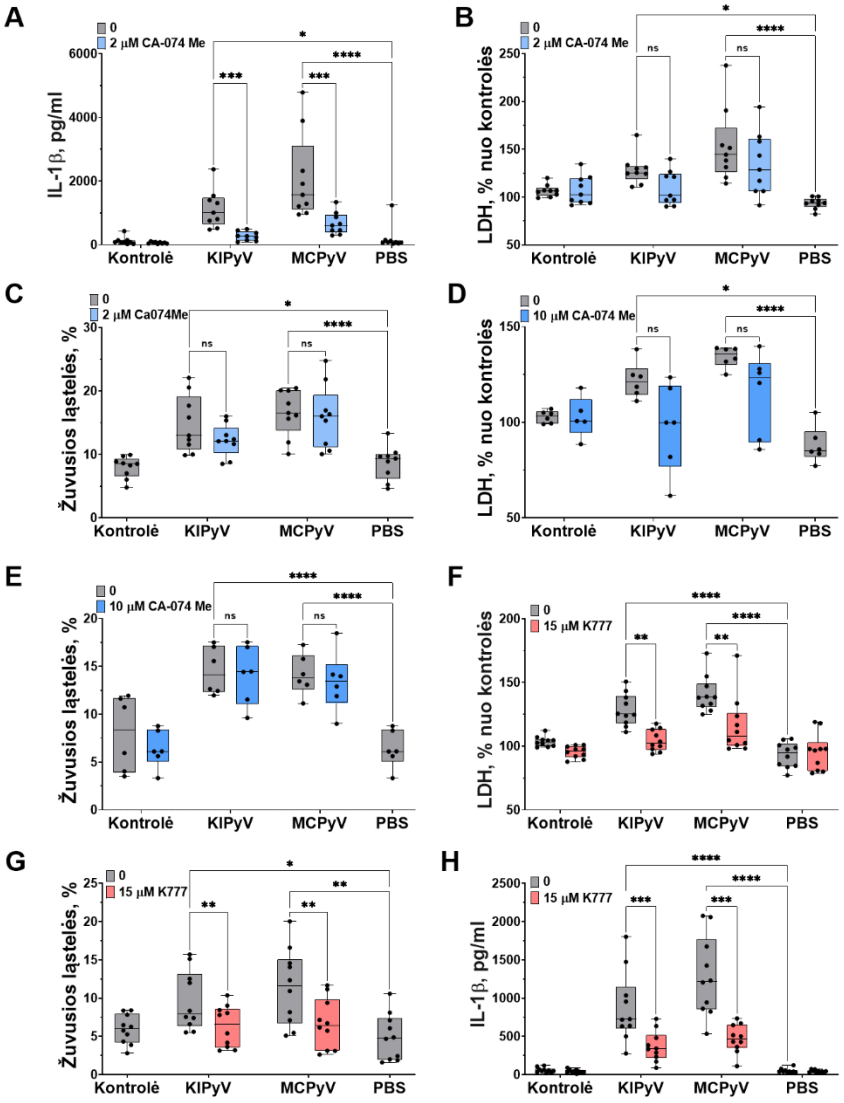


10 pav. PyV VLP sukėlė kaspazės-1 aktyvinimą THP-1 makrofaguose. THP-1 makrofagai buvo veikiami 15 h PyV VLP. "+" – rodo MCC950 poveikį. (A) Aktyvios kaspazės-1 fragmentas p20 buvo nustatytas imunobloto metodu. Imunoblote pavaizduoti eksperimento dublikatai. (B, C) Ląstelės buvo dažomos FLICA reagentu (aktyvi kaspazė-1); branduoliai – PI, Hoechst. (B) Reprezentacinės nuotraukos. Skalė – 100 μm. (C) Aktyvios kaspazės

skaitinis įvertinimas (n = 40 nuotraukų). , n = 1. *p < 0.05, ****p < 0.0001 – pagal *Kruskal–Wallis* testą kartu su *Dunn's post hoc* testą.

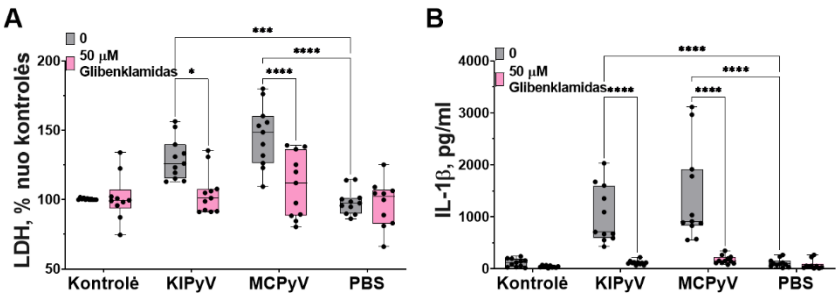
Toliau tyrėme inflamasomos aktyvinimo PyV VLP mechanizmą. Pradėjome nuo mechanizmų, siejamų su fagocituojamomis dalelėmis. Vienas iš mechanizmų yra lizosomų pažaidos (Shu and Shi, 2018). Jų metu į citozolį išskiriamos proteazės katepsinai, kurie inicijuoja signalinę kaskadą, lemiančią inflamasomos aktyvinimą (Rashidi *et al.*, 2020). Šios aktyvios proteazės toliau sukelia signalinę kaskadą, kuri inicijuoja inflamasomos aktyvinimo procesą. Katepsinas B yra vienas iš gerai žinomų veiksnių, dalyvaujančių NLRP3 inflamasomos aktyvinime. Nustatėme, kad šio katepsino slopiklis CA-074 Me reikšmingai sumažino IL-1β sekreciją (Pav. 11A). Tačiau CA-074 Me neturėjo poveikio ląstelių žūčiai (Pav. 11BC). Net ir didesnė CA-074 Me koncentracija nesumažino ląstelių žūties (Pav. 11DE). Tai rodo, kad kiti procesai vyksta inflamasomos aktyvinimo PyV VLP kelyje.

Kitas plačiai naudojamas slopiklis yra plataus spektro katepsinų slopiklis K777. Šis slopiklis sumažino PyV VLP sukeltą ląstelių žūtį (Pav. 12FG) bei IL-1β sekreciją (Pav. 11H). Tai rodo, kad keletas katepsinų dalyvauja inflamasomos aktyvinimo procese. Nors ir matėme statistiškai reikšmingą IL-1β sumažėjimą po poveikio K777, tačiau jis nepasiekė kontrolės lygio. Tai rodo, kad šalia katepsinų kiti veiksniai dalyvauja ląstelių aktyvinimo PyV VLP procese. Literatūroje pateikiami įvairūs duomenys apie inflamasomos aktyvinimo fagocituojamomis dalelėmis mechanizmus. Teigiama, kad nanodalelės aktyvina NLRP3 inflamasomą per lizosomų pažaidas (Hornung *et al.*, 2008). Kiti tyrimai rodo, kad nanodalelių sukeliamas aktyvinimo mechanizmas priklauso nuo jų sudėties ir struktūrinių savybių (Rashidi *et al.*, 2020). Pavyzdžiui, cholesterolio kristalai indukuoja NLRP3 inflamasomą iš dalies nuo katepsinų priklausomu keliu. Katepsinų slopikliai sumažina tik cholesterolio kristalų sukeltą IL-1β sekreciją. Mūsų tyrime taip pat matėme, kad katepsino B slopiklis nuslopino tik IL-1β sekreciją, sukeltą PyV VLP.



11 pav. Katepsino B slopiklis sumažino IL-1β sekreciją, o katepsinų slopiklis (K777) sumažino IL-1β ir ląstelių žūtį THP-1 makrofaguose. THP-1 makrofagai buvo veikiami 24 h PyV VLP. Slopikliai CA-074 Me ir K777 buvo įdėti 30 min prieš VLP poveikį. (A, H) IL-1β sekrecija buvo matuojama supernatantuose ELISA metodu. Citotoksiškumas įvertintas (B, D, F) LDH testu, ir (C, E, G) Branduolių dažymu PI ir Hoechst. n = 6-10, *p < 0,05, **p < 0,01, ***p < 0,001, ****p < 0,0001, – pagal ANOVA, atliekant *Tukey's* testą.

Lizosomų pažaidų metu vykstantys procesai, pavyzdžiui, reaktyvių deguonies formų susidarymas ar lipidų oksidacija, gali sukelti inflamasomos aktyvinimą (Wang *et al.*, 2018a). Vienas iš tyrimų parodė, kad porų susidarymas lizosomų membranoje skatina K^+ jonų išskyrimą ir $Ca2^+$ jonų patekimą į ląstelę, o tai sukelia NLRP3 inflamasomos aktyvinimą (Katsnelson *et al.*, 2016). Dėl to pasirinkome kitą slopiklį – glibenklamidą, kuris slopiną K^+ jonų išskyrimą iš ląstelės. Glibenklamidas reikšmingai sumažino PyV VLP sukeltą ląstelių žūtį (Pav. 12A) ir IL-1β sekreciją (Pav. 12B). Taigi, parodėme, kad PyV VLP aktyvina inflamasomą sudėtiniu mechanizmu, susidarant lizosomų pažaidoms, kurių metu išskiriami katepsinai, ir vykstant K^+ jonų išskyrimui iš ląstelių. Ankstesni tyrimai su kitais oligomeriniais baltymais – prionais – parodė, kad NLRP3 inflamasomos aktyvinimas vyksta dėl K^+ jonų koncentracijos pokyčio po lizosomų pažeidimo (Hafner-Bratkovič *et al.*, 2012).



12 pav. K+ jonų išskyrimo iš ląstelės slopiklis sumažino PyV VLP sukeltą THP-1 makrofagų aktyvinimą. THP-1 makrofagai buvo veikiami 24 h PyV VLP. Slopiklis glibenklamidas buvo pridėtas 30 min prieš VLP poveikį. (A) Citotoksiškumas įvertintas LDH testu. (B) IL-1β sekrecija buvo matuojama supernatantuose ELISA metodu. n = 11, *p < 0,05, ***p < 0,001, ****p < 0,0001 – pagal ANOVA, atliekant *Tukey's* testą.

Apibendrinant, parodėme, kad NLRP3 inflamasoma yra aktyvinama mažos molekulinės masės Aβ oligomerais, PyV VLP, bet ne NLP. Inflamasomos aktyvinime fagocituojančiomis dalelėmis dalyvauja įvairūs veiksniai, tarp jų katepsinai, ir vyksta kompleksiniai procesai. Mūsų tyrimas rodo, kad skirtingos kilmės oligomeriniai baltymai, virusiniai ir endogeniniai, sukelia uždegiminį atsaką makrofaguose, priklausomai nuo jų struktūrinių ypatybių. Šie duomenys galėtų būti panaudojami kuriant vakcinas. Jie suteikia naujos informacijos apie inflamasomos aktyvinimo baltyminės kilmės medžiagomis mechanizmus.

IŠVADOS

1. Aβ oligomerai ir protofibrilės aktyvino NLRP3 inflamasomą pelės mikroglijos ląstelėse – tai rodo, kad pirminės amiloidų struktūros gali sukelti neuroužd egiminį atsaką.
2. Mikroglijos ląstelės fagocitavo Aβ oligomerus ir protofibriles vienodai, tačiau abiem atvejais buvo matoma maža ląstelių populiacija, turinti didesnį Aβ kiekį – tai rodo, kad dalis mikroglijos pasižymi aukštesniu fagocitiniu aktyvumu.
3. Filamentinės struktūros tymų ir kiaulytės virusų NLP nesukėlė uždegiminio atsako nei pelės mikroglijos ląstelėse, nei žmogaus THP-1 makrofaguose.
4. Sferines struktūras formuojančios poliomos virusų KIPyV ir MCPyV VLP sukėlė uždegiminį atsaką ir aktyvino NLRP3 inflamasomą žmogaus THP-1 makrofaguose bei pirminiuose žmogaus makrofaguose – tai rodo oligomerinių baltymų struktūros svarbą ląstelių aktyvinimui.
5. Katepsinų ir K+ išėjimo iš ląstelės slopikliai sumažino inflamasomos aktyvinimą KIPyV ir MCPyV VLP – tai rodo, kad inflamasoma yra aktyvinama katepsinais, patekusiais į citozolį lizosomų pažaidų metu, arba dėl šio proceso įvykusiu K+ pašalinimu.
6. Oligomerinių baltymų struktūrinės savybės yra vienas iš pagrindinių veiksnių, nulemiančių makrofagų uždegiminį atsaką.

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1. Lučiūnaitė A, McManus RM, Jankunec M, Rácz I, Dansokho C, Dalgėdienė I, Schwartz S, Brosseron F, Heneka MT. Soluble Aβ Oligomers and Protofibrils Induce NLRP3 Inflammasome Activation in Microglia. J Neurochem. 2019 Dec 23:e14945. doi: 10.1111/jnc.14945.
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4. Lučiūnaitė, A.; Dalgėdienė, I; Žvirblienė, A. "Activation of Macrophages with Oligomeric Proteins and Their Immune Complexes", XIVth International Conference of Lithuanian Biochemical Society, Vilnius, Lithuania, 27-30 June, 2016.

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1. Luciunaite, A.; MacManus R.; Schwartz S.; Jankunec M.; Brosseron F.; Heneka MT. NLRP3 Inflammasome Activation by Amyloid-beta Oligomers in Microglia Cells. International Conference of Life Sciences „The Coins 2020", Vilnius, Lithuania, 25-27 February, 2020.
2. Poster and flash talk: Lučiūnaitė, A.; MacManus R.; Jankunec M.; Schwartz S.; Latz E.; Heneka MT. Soluble Aβ Oligomers Induce NLRP3 Inflammasome Activation in Microglia Cells. EMBO workshop Cell death in immunity and inflammation. Crete, Greece, 6-9 October, 2019.
3. Lučiūnaitė, A.; MacManus, R.; Jankunec, M.; Schwartz, S.; Latz, E.; Heneka MT. Soluble Aβ Oligomers Induce NLRP3 Inflammasome Activation in Microglia Cells. 6th Venusberg Meeting on Neuroinflammation. Bonn, Germany, 9-11 May, 2019.

4. Lučiūnaitė, A.; MacManus, R.; Jankunec, M.; Schwartz, S.; Latz, E.; Heneka MT. Soluble Aβ Oligomers Induce NLRP3 Inflammasome Activation in Microglia Cells. 5th BonnBrain Meeting. Bonn, Germany, 25-27 March, 2019.
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Awards	• Travel grant for conference „EMBO workshop: Cell death in immunity and inflammation" from EMBO (2019) and from Research Council of Lithuania. • One year promotional doctoral scholarship from Research Council of Lithuania (2019). • Scholarship grant for internship at University Hospital Bonn from Research Council of Lithuania, project No.: 09.3.3-LMT-K-712-14-0019 (05/2019-04/2019). • Scholarship grant for internship at University Hospital Bonn from Lithuanian Education Exchanges Support Foundation (01/2019-09/2018). • One-year Erasmus+ scholarship for internship at University Hospital Bonn (Universitätsklinikums Bonn) (04/2017-04/2018). • Travel grant for conference „EMBO/EMBL symposium: Mechanism of Neurodegeneration" attendance from EMBL Advanced Training Centre (2017). • Winner prize of 3rd place for the best oral presentation at Lithuanian Academy of Sciences conference "Bioateitis" (2016).

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