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DOKUZ EYLUL UNİVERSİTY

IZMIR INTERNATIONAL BIOMEDICINE AND GENOME INSTITUTE

IN VIVO AND *IN VITRO* EFFECTS OF BILIRUBIN IN NLRP3 INFLAMMASOME ACTIVATION

İLKCAN ERCAN ORHAN

MOLECULAR BIOLOGY AND GENETICS

Ph.D. THESIS

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LIST OF ABBREVIATIONS

ABE: Acute Bilirubin Encephalopathy

AIM2: Absent in Melonoma 2

ASC: Apoptosis-Associated Speck-Like Protein Containing Caspase RecruitmentDomain

ATP: Adenosine Tri-Phosphate

BBB: Blood-brain Barrier

BIND: Bilirubin--Induced Neurological Dysfunction Ca2+:

Calcium IonsCaspase-$1^{-/-}$ Caspase-1 knock-outCBE:

Chronic Bilirubin Encephalopathy

cDNA: Complementary DNA

Cl: Chloride Ions

CNS: Central Nervous System

CSF: Cerebrospinal Fluid

DAMPS: Danger Associated Molecular Patterns

ELISA: Enzyme-Linked ImmunoSorbent Assay

FBS: Fetal Bovine Serum

GAPDH: Glyceraldehyde 3-phosphate DehydrogenaseHCl:

Hydrochloric Acide

HIF 1: Hypoxia-inducible Factor 1

HRP: Horseradish Peroxidase IKK:

Inhibitor of NF-κB Kinase

IL-1β: Interleukin-1 beta

IL-18: Interleukin 18

IRAK-1: IL-1 Receptor Associated Kinase 1

K+: Potassium ions

LDH: Lactate Dehydrogenase Assay

LPS: Lipopolysaccharide

LRR: Leucine-rich Repeat

MAPK: Mitogen-activated Protein Kinase

NaOH: Sodium Hydroxide

mRNA: Messenger RNA NF-

κB: Nuclear Factor-κB NLRs:

NOD-like Receptors

NLRP1: NOD-like Receptor Protein 1

NLRP3: NOD-Like Receptor Protein 3

NLRC4: NOD-Like Receptor Protein 4

$Nlrp3^{-/-}$: NLRP3 knock-out

NO: Nitric Oxide

NOD2: Nucleotide-binding Oligomerization Domain-Containing Protein 2PI:

Propidium Iodide

PRRs: Pattern Recognition Receptors

PAMPS: Pathogen Associated Molecular Patterns

PYHIN: Pyrin and HIN domain-containing Protein

qPCR: Quantitative Polymerase Chain Reaction SEM:

Standard Error of the Mean

siRNA: Small-interfering RNA

SDS-PAGE: Sodium Dodecyl Sulphate polyacrylamide gel electrophoresisSOD:

Superoxide Dismutase

PVDF: Polyvinylidene Difluoride

TLRs: Toll-like Receptors

TNF: Tumor Necrosis Factor

PYD: Pyrin Domain

RLRs: RIG-I-like receptors

UCB: Unconjugated Bilirubin

Ugt-1: UDP Glucuronosyltransferase 1 Family

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IN VIVO AND *IN VITRO* EFFECTS OF BILIRUBIN IN NLRP3 INFLAMMASOME ACTIVATION

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ABSTRACT

Despite contemporary improvements in neonatal care, bilirubin-induced neurological dysfunction (BIND), which is caused by hyperbilirubinemia, continues to be a major cause of death or permanent disability in the developing world. Inflammationis thought to be one of the main causes of brain damage after bilirubin exposure, eventhough the precise mechanism is still unknown. This project aims to investigate the effect of bilirubin in inflammasome activation and pyroptotic cell death in *in vitro* and *in vivo* examinations and to decide if the inflammasome activation depends on NOD-Like Receptor 3 (NLRP3. 50 µM bilirubin administration in N9 microglia cell line results in inflammasome activation assessed by increased IL-1β cytokine, IL-1β, NLRP3 mRNA expression and caspase-1 (p20), IL-1β and NLRP3 protein level. The relation betweenbilirubin-related inflammasome activation leading to microglial cell death was successfully determined by propidium iodide staining. Whether inflammasome activation and cell death are mediated via the NLRP3 pathway was confirmed by functional assays with NLRP3 siRNA inhibition. Following injection of bilirubin into the *cisterna magna* of a neonatal mouse, activation of the NLRP3 inflammasome was determined by elevated IL-1β, NLRP3 mRNA, and protein level and caspase-1(p20) protein secretion in wild-type animals. On the contrary, bilirubin injected NLRP3 knock-out (*Nlrp3*$^{-/-}$) and caspase-1 knock-out (*Caspase-1*$^{-/-}$) mice has not released active caspase-1 or IL-1β. All of these findings suggest that bilirubin plays a part in the activation of the inflammasome in the brain and that NLRP3 is essential for this activation. With the identification of underlying molecular pathways, additional researchon the connection between the NLRP3 inflammasome and bilirubin toxicity mayprovide a new direction for the development of novel therapeutic approaches in bilirubin toxicity .

BİLİRUBİNİN NLRP3 İNFLAMAZOM AKTİVASONU ÜZERİNE *İN VİVO* VE *İN VİTRO* ETKİSİ

İlkcan Ercan Orhan, İzmir Uluslararası Biyotıp ve Genom Enstitüsü, Dokuz EylülÜniversitesi, İzmir, Türkiye

ÖZET

Yenidoğan bakımındaki çağdaş gelişmelere rağmen, hiperbilirubineminin neden olduğu bilirubin kaynaklı nörolojik disfonksiyon (BIND), gelişmekte olan ülkelerde önemli bir ölüm veya kalıcı sakatlık nedeni olmaya devam etmektedir. İnflamasyonun,bilirubin maruziyetinden sonra beyin hasarının ana nedenlerinden biri olduğu düşünülmektedir, ancak kesin mekanizma hala bilinmemektedir. Bu projenin amacı, *in vitro* ve *in vivo* incelemelerde bilirubinin inflamatuar aktivasyon ve piroptotik hücre ölümünün etkisini araştırmak ve inflamatuar aktivasyonun NLRP3'e bağlı olup olmadığına karar vermektir. N9 mikroglia hücre hattında 50 μM bilirubin uygulamasısonucu, IL-1β sitokin artışı, IL-1β, NLRP3 mRNA ekspresyonunda artış ve kaspaz-1 (p20), IL-1β ve NLRP3 protein seviyelerinde yükselme ile inflamazom aktivasyonu olduğu gösterilmiştir. Mikroglial hücre ölümüne yol açan bilirubin ile ilişkili inflamazom aktivasyonu arasındaki ilişki propidium iyodür boyama ile belirlemiştir. İnflamazom aktivasyonu ve hücre ölümünün NLRP3 yolu aracılığıyla aracılık edip etmediği, NLRP3 siRNA inhibisyonu ile fonksiyonel analizlerle doğrulanmıştır. Yenidoğan yabanıl tip farelerde cisterna magnaya 10 μg ve 20 μg bilirubin enjeksiyonunu takiben IL-1β, NLRP3 mRNA ve protein seviyelerinde artış, kaspaz-1 (p20) salgılanmasında artış ile NLRP3 inflamazom aktivasyonu gösterildi. Aksine, bilirubin enjekte edilen NLRP3 nakavt (*Nlrp3*$^{-/-}$) ve kaspaz-1 nakavt (*Kaspaz-1*$^{-/-}$) fareleri aktif kaspaz-1 veya IL-1β salgılamamıştır. Tüm bu bulgular, bilirubinin beyindeki inflamazom aktivasyonunda rol oynadığını ve NLRP3'ün bu aktivasyon için gerekli olduğunu göstermektedir. Altta yatan moleküler yolakların tanımlanmasıyla, NLRP3 inflamazom ve bilirubin toksisitesi arasındaki bağlantı üzerine ek araştırmalar, bilirubin toksisitesinde yeni terapötik yaklaşımların geliştirilmesi için yeni bir yön sağlayabilir.

1. INTRODUCTION AND AIM

1.1. Statement and Importance of the Problem

While bilirubin is mostly bound to albumin, it can occasionally be present in the central nervous system as free bilirubin, which can damage the nervous system's development. Exposure sustained at high levels of unconjugated bilirubin (UCB) may cause irreversible cellular damage. Hyperbilirubinemia may lead to inflammation and excitotoxicity. Inflammasome activation is a process that has a role in the initiation of inflammatory responses. Membrane-bound or cytosolic receptors are involved ininflammasome activation. One of these is NOD-like receptors (NLRs). It ends with theactivation of caspase-1 and the release of interleukin IL-1β from the cell resulting in cell death called pyroptosis. The molecular mechanism of bilirubin neurotoxicity has not yet been fully illuminated. Because neuroinflammation takes part in bilirubin-related neurological dysfunction (BIND) pathogenesis, there has been no information about the effect of bilirubin on NLRP3 inflammasome. Thus, identifying the molecular mechanisms of bilirubin neurotoxicity and potential new therapeutic approaches will come from investigating the relationship between inflammasome and bilirubin.

1.2. Aim of the Study

The aims of the thesis are;

1) To investigate the effect of bilirubin in inflammasome activation N9 microglial cellline
2) Determine if the inflammasome activation that triggers by bilirubin is related NLRP3 *in vitro*
3) *In vivo* determination of bilirubin effect on inflammasome activation
4) To verify if the inflammasome activation due to bilirubin in neonates is mediated with NLRP3 by using *Nlrp3*$^{-/-}$ and *Caspase-1*$^{-/-}$ mouse.

1.3. Hypothesis of the Study

Bilirubin induces NLRP3 inflammasome activation and cell death in N9 murine microglial cells and neonatal mouse brains.

2. GENERAL INFORMATION

2.1. Bilirubin Metabolism

Bilirubin is a tetrapyrrole molecule, consisting of four pyrrole rings linked by carbon bridges. Its chemical formula is C33H36N4O6 with a cyclic structure (Kang, Lee et al. 2014) **(Figure 1)**. Bilirubin is the yellowish substance that gives bile itscolor. It is revealed by the destruction (breakdown) of an organic molecule called heme. Heme is part of a protein called hemoglobin inside erythrocytes, which are cells that carry oxygen in the blood. Each hemoglobin contains 4 hemes. Heme contains an iron (Fe) in the middle and oxygen is connected to the iron here. When old erythrocytesare broken down, heme turns into bilirubin. There are 5 steps in normal bilirubin metabolism; 1) production, 2) uptake by hepatocytes, 3) conjugation, 4) excretion, and

5) degradation and delivery to the intestine. A problem with any of these steps in bilirubin metabolism can lead to an excess of bilirubin in the blood, a condition known as hyperbilirubinemia. Hyperbilirubinemia can have several causes, including liver disease, red blood cell disorders, and problems with the bile ducts. (Gartner and Arias 1969, Ansong-Assoku, Shah et al. 2022, Kalakonda, Jenkins et al. 2022)

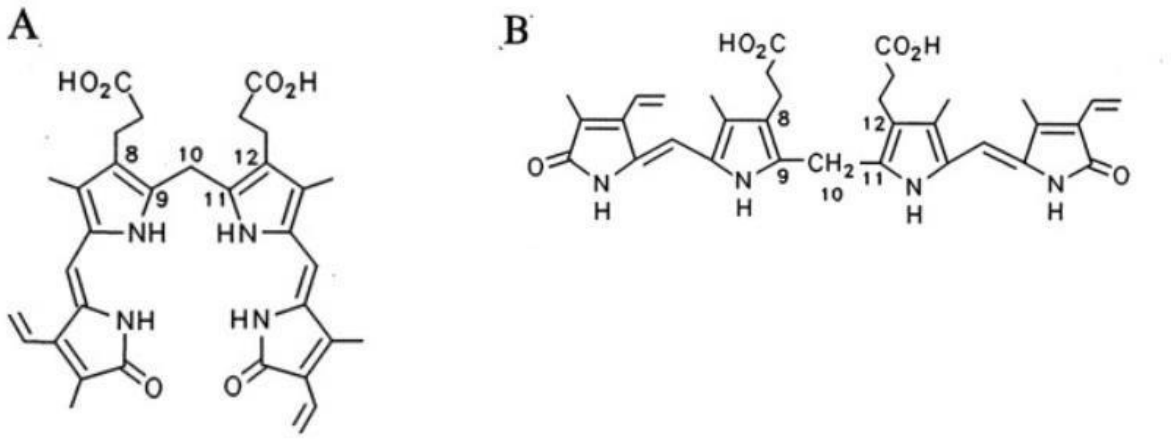

Figure 1: (A) porphyrin-like and (B) linear representations of bilirubin (Nogalesand Lightner 1995) .

Indirect bilirubin (free bilirubin, unconjugated bilirubin) is the first type of bilirubin to form after the liver starts working. It's known as free bilirubin because it's unbound from other substances in the blood. Indirect bilirubin is not soluble in water and does not go into the urine so not excreted with bile. However indirect bilirubin is liposoluble, meaning it can easily pass through membranes and diffuse into tissues (Ahlfors 2001).Indirect bilirubin reacts with UDP-glucuronic acid, which is formed in the path of the breakdown of glucose through glucuronic acid in a reaction catalyzed by the enzyme UDP-glucuronyl transferase, a microsomal enzyme in the flat endoplasmic reticulum microsomes of hepatocytes, and is conjugated with glucuronic acid, thus forming direct bilirubin (conjugated bilirubin). Indirect bilirubin, which occurs in cells of the extrahepatic reticuloendothelial system, is transported to the liver through the circulation, usually binding to albumin (Kang, Lee et al. 2014, Hansen, Wong et al. 2020).

2.2. Neonatal Hyperbilirubinemia

Neonatal hyperbilirubinemia is a condition in which there is an excessive buildupof bilirubin in the blood of newborn infants. According to observational research,

hyperbilirubinemia is thought to affect 60% of term and 80% of preterm newborns. Symptoms may include jaundice (yellowing of the skin and whites of the eyes), lethargy, and poor feeding. It is usually a mild, temporary, and self-limiting condition known as "physiological jaundice," which goes away on its own without therapy. It is crucial to differentiate this from a more severe variant known as "pathological jaundice," though. Hyperbilirubinemia can be treated with phototherapy (exposure to special lights) or, in severe cases, with a blood transfusion. It is important to monitor and treat hyperbilirubinemia promptly to prevent complications such as brain damage(Abbey, Kandasamy et al. 2019, Qian, Kumar et al. 2022)

Jaundice in the newborn period is examined under two headings as indirect and direct hyperbilirubinemia.

2.2.1 Conjugated Hyperbilirubinemia

A type of hyperbilirubinemia where there is an excess of direct or conjugated bilirubin in the blood. It occurs when there is a problem with the liver's ability to excretebilirubin into the bile ducts, causing it to build up in the blood. This type of hyperbilirubinemia can be a sign of liver disease (Tripathi and Jialal 2022) . The causesof conjugated hyperbilirubinemia can be broadly divided into two categories: intrahepatic and extrahepatic. Intrahepatic causes refer to problems within the liver, such as liver diseases (hepatitis, cirrhosis) and inherited liver enzyme defects (Gilbert'ssyndrome, Crigler-Najjar syndrome). Extrahepatic causes refer to problems outside ofthe liver, such as blockages in the bile ducts (cholestasis), tumors, or inflammation (Abbey, Kandasamy et al. 2019).

2.2.2 Unconjugated Hyperbilirubinemia

Unconjugated hyperbilirubinemia is the more prevalent type, can be either pathological or physiological. It is a condition in which e excess of unconjugated (indirect) bilirubin in the blood level too high. The etiology of unconjugated hyperbilirubinemia can be divided into three groups based on the mechanism of bilirubin elevation: (Memon, Weinberger et al. 2016, Fargo, Grogan et al. 2017)

1. Increased production: Immune mediated hemolysis like Rhesus and ABO incompatibility or non-immune mediated hemolysis such as red blood cell enzyme defects like glucose-6-phosphate dehydrogenase (G6PD) deficiency or pyruvate kinase deficiency of red blood cells leads to an increase in bilirubin production, which overwhelms the body's ability to clear it.

2. Decreased conjugation: The liver's inability to conjugate bilirubin due to liver disease or inherited disorders like Gilbert's syndrome and Crigler-Najjar syndrome results in a buildup of UCB in the bloodstream

3. Reduced excretion: Decreased excretion of bilirubin from the body through the bile due to liver disease or biliary obstruction leads to an accumulation of UCB in the bloodstream.

Because UCB is lipophilic, it can easily bind to membrane lipids and can easily cross the blood brain barrier (BBB). When it reaches high levels in serum, it has a toxiceffect on the brain. In spite of the tight junctions between capillary endothelial cells thatconstruct the BBB, increased free bilirubin is pass from blood to brain by passive or facilitated diffusion to balance the concentration gradient (Brito, Palmela et al. 2014).

In infants, when indirect bilirubin in plasma is higher than 20-25 mg, it crosses theblood brain barrier and passes to the central nervous system, and may cause bilirubin induced neurological dysfunction (BIND) and even a neuropathic picture called kernicterus as the exposure time is prolonged (Porter and Dennis 2002).

2.3. Bilirubin Neurotoxicity

Terms such as acute bilirubin encephalopathy (ABE), chronic bilirubinencephalopathy (CBE), kernicterus and bilirubin-induced neurological dysfunction (BIND) are used to describe bilirubin neurotoxicity. The American Academy of Pediatrics (AAP) recommended in 2004 that the term "kernicterus" be used for the chronic and permanent clinical sequelae of bilirubin neurotoxicity and "ABE" for the bilirubin toxicity findings seen in the first week after birth to avoid confusion.(Qian, Kumar et al. 2022). In recent years, it has been suggested to use the term bilirubin- induced neurological dysfunction (BIND) to describe the changes associated with bilirubin encephalopathy. BIND shows a wide spectrum of mild and ambiguous

neurological disorders (isolated auditory neuropathy, movement disorders, dystonia, cognitive disorders, mild mental retardation) and acute bilirubin encephalopathy (Shapiro 2005, Johnson and Bhutani 2011)

2.3.1. Bilirubin Induced Neurological Dysfunction (BIND)

A spectrum of neurological damage brought on by acute or chronic exposure of the central nervous system (CNS) to bilirubin is known as bilirubin-induced neurologic dysfunction (BIND). Because it is challenging to define the incidence of CNS dysfunction that may be mild, temporary, and localized, the prevalence of BIND is not well described in the literature (Bhutani and Stevenson 2011). Johnson et al. developed the BIND scoring system that determines the severity of clinical evidences(Johnson, Brown et al. 1999). BIND is a term for bilirubin toxicity that is less severe than kernicterus and has only a mild neurological dysfunction. Bilirubin is mostcommonly stored in the basal ganglia in the brain, affecting the central and peripheralauditory and visual pathways, hippocampus, diencephalon, subthalamic nuclei, midbrain, cerebellum and parts of the brain stem related to oculomotor function, respiratory, neurohumoral and electrolyte control (Bhutani and Wong 2013). This complication is characterized by subtle impairments in hearing, speech, cognition and language, neuromotor function as well as abnormalities in muscle tone. Other signs of this condition include hyperactive neonatal reflexes and a range of neurobehavioral manifestations (Bhutani and Johnson-Hamerman 2015).

2.3.2. Acute Bilirubin Encephalopathy (ABE)

In acute bilirubin encephalopathy, 15% of affected infants may not have significant neurological pathology. Lethargy, hypotonia, and decreased sucking are symptoms of ABE. The disease can still be reversed at this point. BIND numeric scoring systems have been used and validated to assess the severity of ABE. However, if ABE worsens, patients may experience irreversible chronic bilirubin encephalopathy/kernicterus (Qian, Kumar et al. 2022).

2.3.3. Chronic bilirubin encephalopathy (CBE) / Kernicterus

Chronic bilirubin encephalopathy (CBE) and kernicterus are two related but distinct medical conditions. Chronic bilirubin encephalopathy is a broad term for neurological problems caused by elevated bilirubin levels, while kernicterus specifically refers to brain damage in newborns resulting from elevated bilirubin levels. CBE refers to a long-standing accumulation of bilirubin in the brain, which can result in neurological problems such as intellectual disability, spasticity, and ataxia (Connolly and Volpe 1990, Qian, Kumar et al. 2022).

Although there is a high rate of jaundice in the neonatal population, chronic bilirubin encephalopathy is rare disease and kernicterus is a relatively uncommon condition in developed nations but it is relatively more common in underdeveloped regions of the world (Helal, Ghany et al. 2019).

Apoptosis, iron transport, DNA, protein, and neurotransmitter synthesisimpairments, as well as decreased enzyme activity, are all indicators of cellular pathology. Chronic bilirubin encephalopathy is caused by the irreversible and permanent changes brought on by mitochondrial degeneration and brain cell membrane modifications. (Karimzadeh, Fallahi et al. 2020)

2.4. Inflammasome

An inflammasome is a multi-protein complex that plays a key role in the immune system's response to pathogenic infections and other cellular stressors. It activates an innate immune response by cleaving pro-inflammatory cytokines and activating immune cells such as macrophages and dendritic cells. Inflammasomes can be activated by a variety of stimuli, including pathogen-associated molecular patterns (PAMPS), danger-associated molecular patterns (DAMPs) that are recognized by pattern-recognition receptors (PRRs) (Man and Kanneganti 2015). This recognition triggers an immune response, leading to the elimination of the invading microorganisms. PRRs include Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs), among others. Inflammasomes play a crucial role in host defense against infection and are also involved in the development of chronic inflammatory diseases such as gout, atherosclerosis, and certain neurological

disorders. There are several types of inflammasomes, including: NLRP1 (NOD-like receptor protein 1), NLRP3 (NOD-like receptor protein 3), AIM2 (Absent in melanoma 2), NLRC4 (NOD-like receptor protein 4), PYHIN (pyrin and HIN domain-containing protein) (Song and Li 2018).

2.4.1 NLRP3 Inflammasome

In contrast to other inflammasomes, the NLRP3 inflammasome has undergone extensive research and study because it is involved in a variety of diseases. NLRP3 isa member of the NLR protein family, where 22 of its members are found in humans and 34 are known to exist in mice (He, Hara et al. 2016). The NLRP3 inflammasome is composed of several proteins, including a sensor NLRP3, the adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and effector pro-caspase-1. NLRP3 is a cytosolic protein that contains a centerNOD-like domain, amino-terminal pyrin domain (PYD), and carboxy-terminal a leucine- rich repeat (LRR) domain. Pyrin domain interacts with the pyrin domain of ASC to actsas a link between NLRP3 and pro-caspase-1, bringing the two proteins into close proximity to facilitate the activation of caspase-1 (Swanson, Deng et al. 2019).

2.4.2 NRLP3 inflammasome activation

The majority of bacterial-derived stimuli, such as TLR ligands, act to prime inflammasome activation rather than directly activating NLRP3 inflammasome. Additionally, Adenosine tri-phosphate (ATP) and pore-forming toxins have the power to activate NLRP3. As a result, it was proposed that NLRP3 inflammasome activation had two signals: the first signal, known as "priming," and the second signal, known as "activation." (He, Hara et al. 2016, Swanson, Deng et al. 2019)

There are at least two purposes for priming. As its primary function, the inflammasome upregulates the expression of the molecules NLRP3, caspase 1, and pro-IL-1β. The recognition of PAMPs or DAMPs that activate PRRs like Toll-like receptors (TLRs) or nucleotide-binding oligomerization domain-containing protein 2 (NOD2) or through cytokines like tumor necrosis factor (TNF) and IL-1β that trigger

nuclear factor-κB (NF-κB) activation and gene expression can induce this transcriptional upregulation (Bauernfeind, Horvath et al. 2009). Both pro-IL-1β, which is not constitutively expressed in resting macrophages, and NLRP3, which is thought to exist at concentrations insufficient for starting inflammasome activation under resting conditions, are upregulated by NF-κB. But ASC, pro-caspase-1, and pro-IL-18 expression levels do not seem to be impacted by priming signals (Bauernfeind, Horvath et al. 2009, Franchi, Eigenbrod et al. 2009). Furthermore, priming with the TLR4 ligand lipopolysaccharide (LPS) causes a switch in the metabolism of macrophages from oxidative phosphorylation to glycolysis, which in turn results in the stabilization of hypoxia-inducible factor 1 (HIF1) and an increase in IL1B gene transcription.(Swanson, Deng et al. 2019) In addition, recent research has recognized the priming signal's transcription-independent functions. Acute priming with LPS stimulation increases NLRP3 inflammasome activation in a rapid priming procedure even in the absence of NLRP3 induction (Juliana, Fernandes-Alnemri et al. 2012). This quick transcription-independent priming is mediated by the signaling molecule IL-1 receptor associated kinase 1 (IRAK-1), downstream of TLRs and MyD88. It appears that IRAK-1's function in promoting inflammasome activation is not dependent on downstream NF-κB signaling because LPS-induced phosphorylation of IRAK-1 endorses inflammasome activation in a way that is independent of the inhibitor of NF-κB kinase (IKK) complex(Fernandes-Alnemri, Kang et al. 2013, Kelley, Jeltema et al. 2019).

A second step follows the recognition of an NLRP3 activator and causes full activation and inflammasome formation, whereas the priming step of inflammasome activation only licenses the cell. Following this priming stage, a variety of stimuli, such as ATP, pathogen-associated RNA, potassium ions (K+) ionophores, bacterial and fungal toxins and components, particulate matter, and heme, can activate NLRP3. Many upstream signals, most of which are not mutually exclusive, are thought to contribute to NLRP3 activation. These signals include the efflux of K+ or chloride ions (Cl), the flux of calcium ions (Ca2+), mitochondrial dysfunction, lysosomal disruption, trans-Golgi disassembly and metabolic changes (He, Hara et al. 2016) . With the help of these additional signals on immune and inflammatory cells, activated NLRP3 sensor protein to the adaptor protein binds ASC. This interaction leads to the formation of a

platform called the "ASC speck" which recruits and activates the pro-caspase-1enzyme.

Once activated, caspase-1 cleaves pro-IL-1β and pro-IL-18 into their mature forms, which are then released from the cell and contribute to the initiation and amplification of the inflammatory response **(Figure 2).** The NLRP3 inflammasome activation is tightly regulated to prevent excessive inflammation and tissue damage (Shao, Cao et al. 2018)

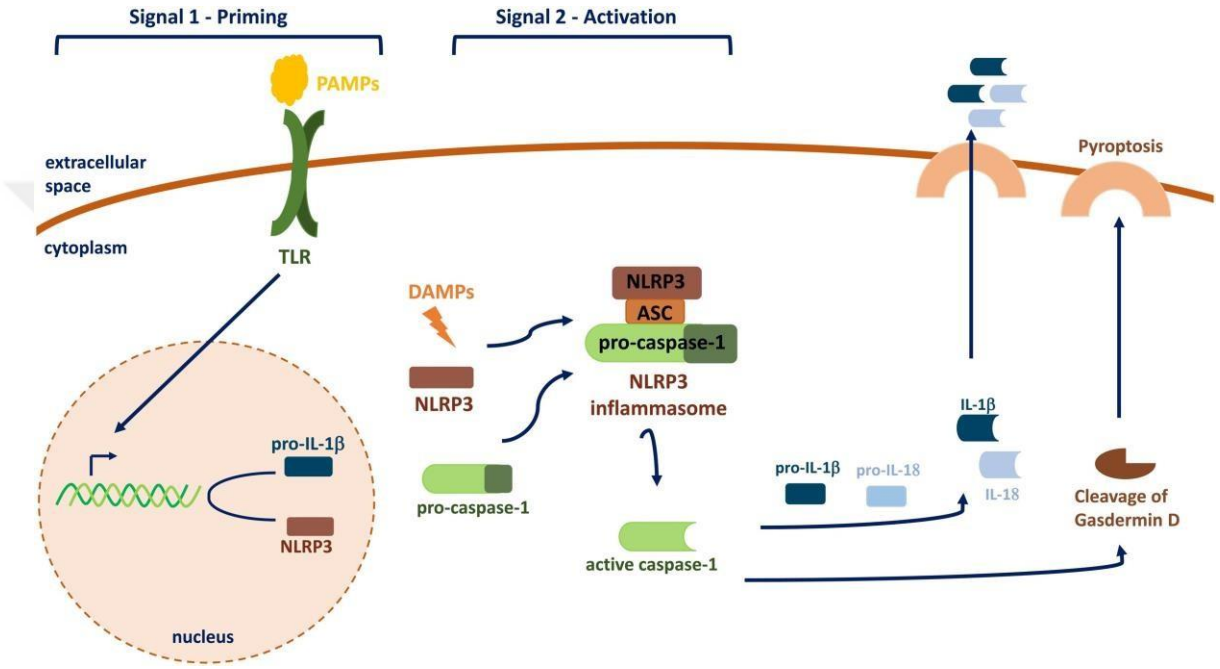

Figure 2: NLRP3 inflammasome activation. PAMPs and DAMPs both work to activate the NLRP3 inflammasome complex. When the inflammasome complex is activated, Caspase-1 in the complex cleaves the precursor of IL-1β and IL-18 in the cell, producing mature IL-1β and IL-18 that are then secreted from the cell. The inflammasome complex is made up of the proteins NLRP3, ASC, and Caspase-1. Additionally, through Gasdermin D, activated Caspase-1 protein may cause pyroptoticcell death (Kiser, Gonul et al. 2021)

2.5. Microglia

The brain is highly complex and specialized structure with different regions with different specific cells. The central nervous system known as a immunologically isolated due to existence of blood brain barrier for many years (Almolda, Gonzalez et al. 2015). However, it is now known that the BBB may permit the active transport of immune molecules and cells from the periphery into the brain, in addition to having its own resident immune cell population (Fumagalli, Perego et al. 2015) Microglia are a type of immune cell that reside in the brain and spinal cord and make up 10-15% of the cells in the CNS (Mittelbronn, Dietz et al. 2001). They are the smallest and most abundant type of glial cell, which is a non-neuronal cell that provides support and protection to neurons. Microglia play a crucial role in the immune defense of the centralnervous (Hammond, Robinton et al. 2018)

2.5.1 Morphology of microglia and activation

Microglia are derived from myeloid progenitor cells and are closely related to other tissue macrophages (Wolf, Boddeke et al. 2017) . They possess multipleprocesses, which give them a characteristic ramified appearance, and allow them to interact with other cells in the brain. The processes of microglia are highly dynamic, allowing them to change their morphology rapidly in response to stimuli (Vidal-Itriago,Radford et al. 2022). In the resting state, microglia exhibit a highly branched morphology, which allows them to constantly monitor the environment and detect anychanges that may occur in the brain. In response to injury or infection, microglia undergo a process called activation, which involves morphological changes and the release of pro-inflammatory molecules. Activated microglia display a more amoeboid shape, with a larger cell body and fewer processes. This morphology enables them tomove more efficiently through the brain tissue and to engulf and phagocytose debris and pathogens. Activated microglia are also characterized by the upregulation of cell surface markers and the release of inflammatory cytokines and chemokines, which recruit and activate other immune cells (Colonna and Butovsky 2017, Vidal-Itriago, Radford et al. 2022).

2.5.2 Function of microglia

Besides their role in immune surveillance, microglia also participate in the development of the nervous system, including the regulation of neuronal proliferation, differentiation, and survival. They play a critical role in synaptic pruning, a process by which excess or non-functional synapses are eliminated during development and throughout adulthood, allowing for proper neuronal connectivity (Wolf, Boddeke et al. 2017). Microglia also play an important role in regulating inflammation in the CNS. Upon activation, they release pro-inflammatory cytokines and chemokines, which recruit other immune cells to the site of injury or infection. However, excessive or chronic activation of microglia can lead to sustained inflammation and neuronal damage, contributing to the development of neurodegenerative diseases (Block and Hong 2005). Recent studies have also shown that microglia play a role in the modulation of synaptic plasticity, a fundamental mechanism underlying learning and memory. They can influence the function and stability of synapses, as well as participate in the clearance of toxic proteins such as amyloid-beta, which accumulatesin Alzheimer's disease (Paolicelli, Bolasco et al. 2011, Hong, Beja-Glasser et al. 2016).

2.5.3 Inflammasome activation in microglia

Microglial cells are cells of myeloid origin that play a role in the formation of the primary immune response in the central nervous system and express TLRs and NLRs (Hanamsagar, Hanke et al. 2012). It is the first cell type in the central nervous system to be shown to have inflammatory activity. In 2008, amyloid-β was shown to induce NLRP3 inflammasome activation in microglial cells in a caspase-1 and cathepsin B- dependent pathway (Liu, Li et al. 2013). Neuron-microglia coculture studies have shown that microglial inflamasome activation causes neuronal death (Hafner- Bratkovič, Benčina et al. 2012). Involvement of inflammasome activation in the pathogenesis of another neurodegenerative disease has been shown in a transgenic mouse ALS model *in vitro* and *in vivo* (Meissner, Molawi et al. 2010). In this study, it was shown in a cell culture study that mutant superoxide dismutase (SOD) causes inflammasome activation in microglial cells that results in IL-1β release. Caspase-1 or IL-1β mutation slowed the course of disease in SOD-defective mice. These findings

suggest that suppression of inflammasome activation in ALS may be a treatment option. An important data is that chronic stress or glucocorticoid treatments cause an increase in hippocampal inflammasome markers in an animal study (Frank, Hershman et al. 2014). A similar finding is the activation of NLRP3 inflammasome in microglial cells without IL-1β increase in serum or cerebrospinal fluid (CSF) in a chronic unpredictable stress model, which is one of the models of depression(Pan, Chen et al.2014).

2.6. Bilirubin, Inflammation and Microglial Cell Relationship

Neuroinflammation is an innate response to harmful effects and increases the release of proinflammatory cytokines, leading to increased permeability in the BBB. Neuroinflammation is involved in the pathogenesis of BIND and besides its effects considered to be protective at low doses, chronic neuroinflammation is considered a critical risk factor for central nervous system damage in the new-born.

Considering the important roles of microglia which are having role in the process of normal brain development and post-natal development, synaptic pruning and tissuerepair, it is conceivable that they may have important roles in hyperbilirubinemia. However, the roles of microglia cells in the increase of neonatal bilirubin are still not fully elucidated. The first reactions of microglia to UCB are an increase in phagocytosis and the transformation of cell shapes into ameboid (Gordo, Falcao et al. 2006). Levels of caspase-8 (extrinsic pathway), caspase-9 (intrinsic) and effector caspase-3 have been shown to reach a maximum with 6-hour exposure to UCB (Silva, Vaz et al. 2010).In addition, it has been reported that excessive UCB has immunostimulatory effects and induces the activation of microglia in acute or chronic form, increases proinflammatory gene expression and causes TNF-α, IL-1β and IL-6 and glutamate secretion from microglia cells and triggers apoptosis/necrosis (Brites and Vaz 2014). NF-ƙB and Mitogen-activated protein kinase (MAPK) release in the early period of inflammation (before 4 hours) has also been identified as a marker for microglial activation with UCB stimulation (Glezer, Simard et al. 2007). In addition, microglia cells migrate to the damage site by triggering molecules such as ATP and nitric oxide (NO) released from damaged tissue in central nervous system damage (Kurpius, Nolley et

al. 2007). ATP has been shown in previous studies to be released from neurons incubated by UCB and NO from other central nervous system cells (Genc, Genc et al.2003, Silva, Vaz et al. 2012). In response to bilirubin-associated toxic damage, proinflammatory cytokines as well as metalloproteinases have been shown to be released from microglia and microglial cells are switched to phagocytic phenotype (Silva, Vaz et al. 2010).

3. MATERIALS AND METHODS

3.1. Type of Research:

This is an *in vitro* and *in vivo* experimental research.

3.2. Time and Place of The Research:

All experimental studies was generally conducted at Izmir Biomedicine and Genome Center between 2019-2022.

3.3. Research Material

N9 microglial cell line developed by Dr. Paola Ricciardi-Castagnoli was used for *in vitro* studies. For the *in vivo* studies C57BL/6 mice were used for both wild type and for background of knock-out animals. NLRP3 knock-out (*Nlrp3*$^{-/-}$, B6.129S6-NLRP3<TM1BHK>/J HOM) and Caspase-1 knock-out (*Caspase-1*$^{-/-}$, B6N.129S2-CASP1<TM1FLV>/J HOM) mice were obtained from Jackson Laboratories (Bar Harbor, ME).

3.4. Variables of the Study

Bilirubin is the independent variable of the study. The dependent variables in this study are cell death rates, mRNA and protein levels of IL1, NLRP3, and caspase-1 inflammasome parameters, as well as cell death type.

3.5. Tool for Data Collection

*3.5.1 N9 Microglia Cell Culture*In this study, the N9 mouse microglial cell line developed by Dr. Paola Ricciardi-Castagnoli was used. For cell cultureRPMI 1640 culture medium containing 100 U/ml penicillin and 100 µg/ml streptomycin, 10% fetal bovine serum (Fetal Bovine Serum; FBS)

was used. The cells were seed in 75 cm2 culture dishes and multiplied by passage with Trypsin/EDTA at a concentration of 0.05% 3 times a week in a cabinet with laminar air flow (ThermoScientific, USA) and the cells were incubated at 37°C in an incubator containing 5% CO2 (ThermoScientific, USA).

3.5.2 Cell Culture Treatment

Cells treated with bilirubin (5 μM-50 μM) for 6 h to induce inflammasome activation. At the same time, cells incubated with potent NLRP3 stimulus LPS (1μg/ml)together with bilirubin were added to the experimental groups. 4-hour (1 μg/ml) LPSand 1-hour (5 mM) ATP model was administered as a positive control.

Bilirubin was dissolved in 0.01N NaOH with bilirubin at 10 mM concentration and setting the concentrations to 5 μM and 50 μM in the experimental environment, and then the pH value was adjusted to 7.4 with 0.1N HCl. It was given to cells without passing through the filter.

Cells; incubated with bilirubin (5 μM-50 μM) for 6 h to induce inflammasome activation. A 4-hour (1 μg/ml) LPS and 1-hour (5 mM) ATP model was administered as a positive control.

3.5.3 IL-1β Cytokine Determination by Enzyme-Linked ImmunoSorbent Assay (ELISA)

IL-1beta cytokine level determination to determine inflammasome activation in microglial cells was performed in accordance with the procedure of the Sandwich ELISA method kit (ThermoScientific, USA , Catalog No: 88-7013-88). The cells were seeded in 96-well plates with $5x10^4$ cells per well for the experiment. Cells were incubated with 5 μM, 50 μM Bilirubin for 5 hours. LPS for 4 hours (1 μg/ml) and ATPfor 1 hour (5 mM) were used as positive controls. After the experiment, the environments of the cells were collected and ELISA analysis was completed inaccordance with the kit protocol. As a absorbance standard 450 nm and 570nm was used for determining absorbance values using MultiSkan Go spectrophotometer

(Thermo Scientific, USA). IL-1β protein concentrations in cell culture media were determined with the help of the standard curve plotted according to IL-1β protein standards.

3.5.4 Propidium Iodide (PI) Staining

N9 cells (1 × 104/well) were seeded into 96-well plates and incubated overnight. After 6 h treatment with 5 μM and 50 μM bilirubin, 50 μg/ml PI dye (Thermo Fisher Scientific, Waltham, MA, USA) was added to each well and incubated 15 minutes. Using fluorescent microscopy, PI-positive cells from 5 different regions of each well were photographed (Olympus IX-71, Japan). PI-positive cells which are dead cells andtotal cells were counted by ImageJ software (National Institutes of Health, USA) and cell death were calculated by comparing PI positive cells to total cell number.

3.5.5 Quantitative PCR (qPCR)

1x 10^6 N9 microglial cells were seeded on T25 flask and next day 5 μM and 50 μM Bilirubin treatment were carried out for 6 h. At the end of the treatment, total RNAfrom cells was obtained by Nucleospin RNA isolation kit (Macherey-Nagel, Germany). The purity and concentration of RNA were evaluated by spectrophotometric measurement with Nanodrop (). High-Capacity cDNA Reverse Transcription Kit (ThermoScientific, USA) with 2 g RNA was used for the synthesis of complementaryDNA (cDNA). cDNA was obtained by incubating the samples in the 'thermal cycler' for10 minutes in 25°C , 120 minutes in 37°C and 5 minutes in 85°C . After cDNA is obtained, the quantitative PCR is used in the ABI 7500 Fast quantitative PCR device with the GoTaq® qPCR Master Mix (Promega; USA) using according to manufacturerinstructions.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene and mRNA levels were examined using mRNA-specific primers of proinflammatory molecules (IL-1β, NLRP3) **(Table 1)**. Gene expression changes werequantitatively calculated by ΔΔCt method using the Ct values obtained for each sample.

NLRP3	Forward	TGCCTGTTCTTCCAGACTGGTGA
	Reverse	CACAGCACCCTCATGCCCGG
IL-1β	Forward	CACAGCAGCACATCAACAAG
	Reverse	GTGCTCATGTCCTCATCCTG
GAPDH	Forward	ACCACAGTCCATGCCATCAC
	Reverse	TCCACCCTGTTGCTGTA

Table 1. The list of primers and the 5'-3' sequences that were used in the qPCR reactions.

3.5.6 Western Blot

For *in vitro* part of the study, 1x 10^6 N9 microglial cells were seeded on T25 flask and next day 50 μM Bilirubin treatment were carried out for 6 h. Cell lysate and supernatant were separated from microglia by after centrifugation. The supernatant was frozen at 80 °C and protein-free part of the supernatant was removed by lyophilization method (freeze drying). RIPA buffer solution containing protease inhibitors was used for total protein isolation. The cells were kept on ice for 30 minutes and then the protein was obtained by centrifugation for 15 minutes at 15000g. **For *in vivo* part of the study**, cell lysis from brain hemisphere was performed with the help of a homogenizer accompanied by RIPA and Halt inhibitor cocktail. Afterwards, the fragmented tissues kept on the ice for 2 hours and were centrifuged at 12000 rpm to obtain protein from the supernatant. Both cell and tissue protein concentrations were determined by BCA protein analysis method. Same amount of each protein was loaded and separated by 8-15 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Separated proteins were transferred from gel to polyvinylidene difluoride (PVDF) membrane and incubated in the blocking buffer (milk or bovine serum albumin) for 1h. After this procedure, the samples were incubated with NLRP3 antibody for cell lysate at 4℃ overnight and Caspase-1 and IL-1β antibodies for supernatant. Beta-actin antibody was used as the housekeeping gene **(Table 2)**. After the overniht

incubation, the membranes containing the samples were incubated with secondary antibodies marked with horseradish peroxidase (HRP) and washed stages were applied within Tris-buffered saline with 0.1% Tween 20 Detergent (TBST). ECL kit based on chemiluminescence method was used for imaging and band densities were evaluated densitometrical.

Antibody	Provider	Catalogue Number
Anti-NLRP3	Adipogen	Ag-20b-0014-c100
AntiCaspase-1	Adipogen	Ag-20b-0042-c100
Anti-IL-1β	Abcam	ab9722
Anti-Beta Aktin	Abcam	ab8227
Anti-rabbit IgG	Cell Signaling	7074S

Table 2. Information about primary and secondary antibodies is used in the western blot analysis.

3.5.7 NLRP3 siRNA transfection

NLRP3 expression was silenced using NLRP3 siRNA (Dharmacon, Mouse Nlrp3 SMARTpool ON-TARGET siRNA, L-053455-00-0005). N9 cells were seeded with antibiotic-free media and next day, NLRP3 siRNA and scrambled siRNA without NLRP3 sequence as a negative control were transfected into cells using Dharmafect transfection reagent (T-2001-03, Dharmacon, USA) with 10% serum media and incubated for 48 hours. At the end of the transfection period, the cell environment was replaced with 50 μM bilirubin included RPMI medium and incubated 6 h to generate inflammasome activation. Total RNA and protein isolation were performed from transfected cells. To evaluate transfection efficiency, NLRP3 mRNA level was compared with the control siRNA transfected samples. NLRP3 siRNA and control siRNA transfected and 50 μM bilirubin incubated cells were used to determine caspase-1 and IL-1β protein level by following western blot method.

3.5.8 Animals and Model Construction

All animal studies were accomplished according to procedures provided by the Experimental Animal Laboratory and accepted by Izmir International Biomedicine and Genome Institute Local Ethics Committee for knock-out animals (Protocol number: 01/2020) and the Animal Care and Use Committee of the Dokuz Eylul University School of Medicine for wild type mice (Protocol number: 42/2018). For WT studies C57BL/6 mice were used. NLRP3 knock-out (Nlrp3−/−, B6.129S6- NLRP3<TM1BHK>/J HOM) and Caspase-1 knock-out (Caspase-1−/−, B6N.129S2- CASP1<TM1FLV>/J HOM) mice in the C57BL/6 background were obtained from Jackson Laboratories (Bar Harbor, ME). All animals were maintained in the vivarium under controlled conditions (23±2°C; 12 h light/dark periods) with access to food and water ad libitum. Both wild type (WT) (C57BL/6) and *Nlrp3* $^{-/-}$ and *Caspase-* $^{-/-}$ mice were treated with the same procedure to female mice with a period of estrus detectedin the vaginal smear for pregnancy. Mice were monitored with their mothers after birth. When the mice were postnatal 5 days old, the kernicterus model for the hyperbilirunemia group was performed by injection of 10 μg/g (weight) and 20 μg/g (weight) bilirubin via cisterna magna puncture in accordance with the literature. Underthe protection of light, bilirubin (B4126, Sigma-Aldrich, USA) was dissolved in 0.5 M NaOH solution and diluted in ddH2O to 10 mg/ml. HCl (0.5 M) was then used to raisethe pH to 8.5. (Song, Hu et al. 2014). The puppies were anaesthetized using diethyl ether. A similar amount of CSF was removed before bilirubin was given. The Sham group undertook the same procedure and received an equal dose of saline instead ofbilirubin from cisterna magna. Within 24 hours of bilirubin injection, the mice were sacrificed by cervical dislocation and their brain tissues were separated for molecular biological examinations.

3.5.9 Statistical Analysis

Each experiment's data analyses were completed using GraphPad Prism 8.0 (Graphpad Software Inc., CA, USA). The Mann-Whitney U test was used to compare independent groups. The values were given as mean ± standard error of the mean (SEM). Analyses with a p-value less than 0.05 ($p<0.05$) were considered statistically significant.

3.6. Study Plan and Calendar

1.Determination of *in vitro* effect of inflammasome activation	August 2020-January 2021
2. Determination of if the inflammasome activated bybilirubin result from NLRP3 by using *in vitro* knockdown method	January 2021- May 2021
3.Determination of *in vivo* effects of inflammasome activation	May 2021-September 2021
4. *İn vivo* determination of if the inflammasome activated by bilirubin result from NLRP3	September 2021-December 2021

3.7. Limitations of The Study

The first limitations in this thesis is that the study was conducted in the N9 microglia cell line. Although primary microglia cells have similarities with N9 cell lines,it is appropriate to confirm study results in primary microglia cells. Another limitation is the color of the bilirubin. Because colorimetric measurements like LDH or could not performed due to overlap of the absorbances. Finally, it is not known through which pathway bilirubin influences the activation of inflammasomes. These pathways should be examined in future studies.

3.8. Ethics Committee Approval

All animal experiments were carried out in accordance with protocols approvedby the Dokuz Eylul University School of Medicine's Animal Care and Use Committeefor WT mice (Protocol number: 42/2018) and the Izmir International Biomedicine andGenome Institute Local Ethics Committee for knock-out animals (Protocol number: 01/2020). These protocols were supplied by the Experimental Animal Laboratory.

4. RESULTS

4.1. Determination of the relationship of bilirubin with inflammasome by IL-1β cytokine determination

To determine whether bilirubin caused an inflammasome in the N9 mousemicroglial cell line, bilirubin incubation was applied during 6 h. The experimental groups were determined as control, 5 μM bilirubin, 50 μM bilirubin and positive control LPS+ATP. IL-1β cytokine level was examined by ELISA sandwich method and it was found cytokine level was significantly increased in 5 μM, 50 μM bilirubin treated cells and LPS+ATP as positive control treated groups (**Figure 3**)

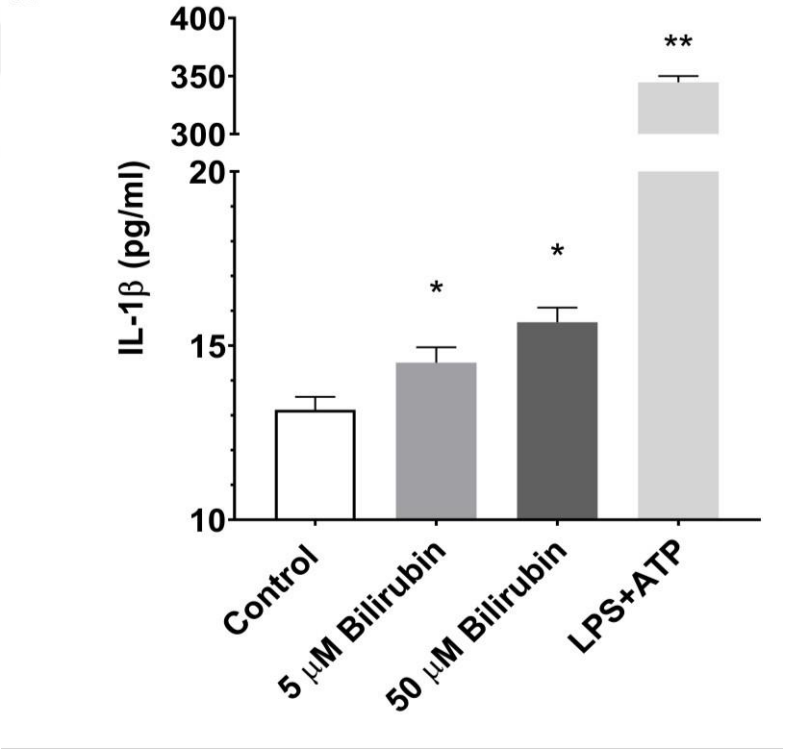

Figure 3: Dose dependent effect of bilirubin on IL-1β cytokine levels were defined to increase significantly with bilirubin treatment. LPS+ATP was used as a positive control. The results are presented as mean ± S.E.M, n = 5. *p<0.05 and
**p<0.01 compared with untreated control.

4.2. Effect of bilirubin on mRNA expression levels IL-1β and NLRP3 genes by qPCR

IL-1β mRNA expression changes were detected in order to highlight the effect ofbilirubin in the transcription of inflammasome markers. qPCR analyses showed that 5 μM and 50 μM bilirubin treatment significantly increased IL-1β mRNA expression level 5,1-fold and 3,8-fold respectively. NLRP3 is the key protein that activatesproinflammatory molecules. Therefore, to understand the bilirubin effect in NLRP3 mRNA expression, mRNA expression level of NLRP3 was measured by q-PCR analysis. 50 μM bilirubin had a significant effect NLRP3 mRNA expression level about1,3-fold. LPS and ATP treatment was used as a positive control and displayed 1333- fold change on IL-1β mRNA expression level and 6,5-fold change on NLRP3 mRNA expression level according to control group **(Figure 4).**

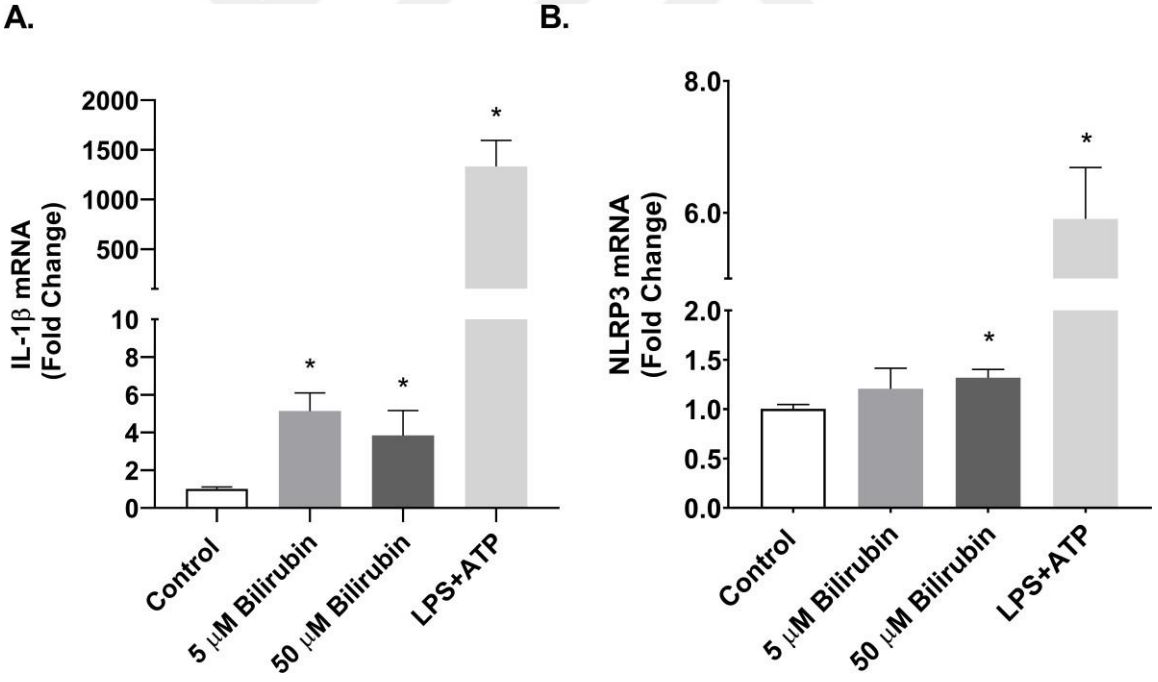

Figure 4: Effects of bilirubin on expression levels of mRNA of indicator genes of inflammasome. (A) IL-1β (B) NLRP3. LPS+ATP used as a positive control. The results are presented as mean ± S.E.M, n = 5. *p<0.05 and **p<0.01 compared with untreated control.

4.3. The effect of bilirubin on pyroptotic cell death induced by inflammasome activation

NLRP3 inflammasome activation causes pyroptotic cell death. The effect of bilirubin on cell death caused by inflammasome activation was demonstrated by LDH and PI staining method in 6h bilirubin treated N9 microglial cells. No significant results were found in LDH release other than the positive control LPS+ATP group. It was concluded that yellowish colored bilirubin could affect the absorbance values and that the LDH method would not be suitable for assessing cell death. Cell death for positive control in PI-stained cells after LPS+ATP incubation was approximately 66%. PI positive cell count increased significantly by 13% in cells incubated with 50 µM Bilirubin, 2% in cells incubated with 5 µM Bilirubin. According to percentages, it was understood that 50 µM bilirubin triggers inflammasome dependent pyroptotic cell death and is not cytotoxic for N9 microglial cells **(Figure 5)**.

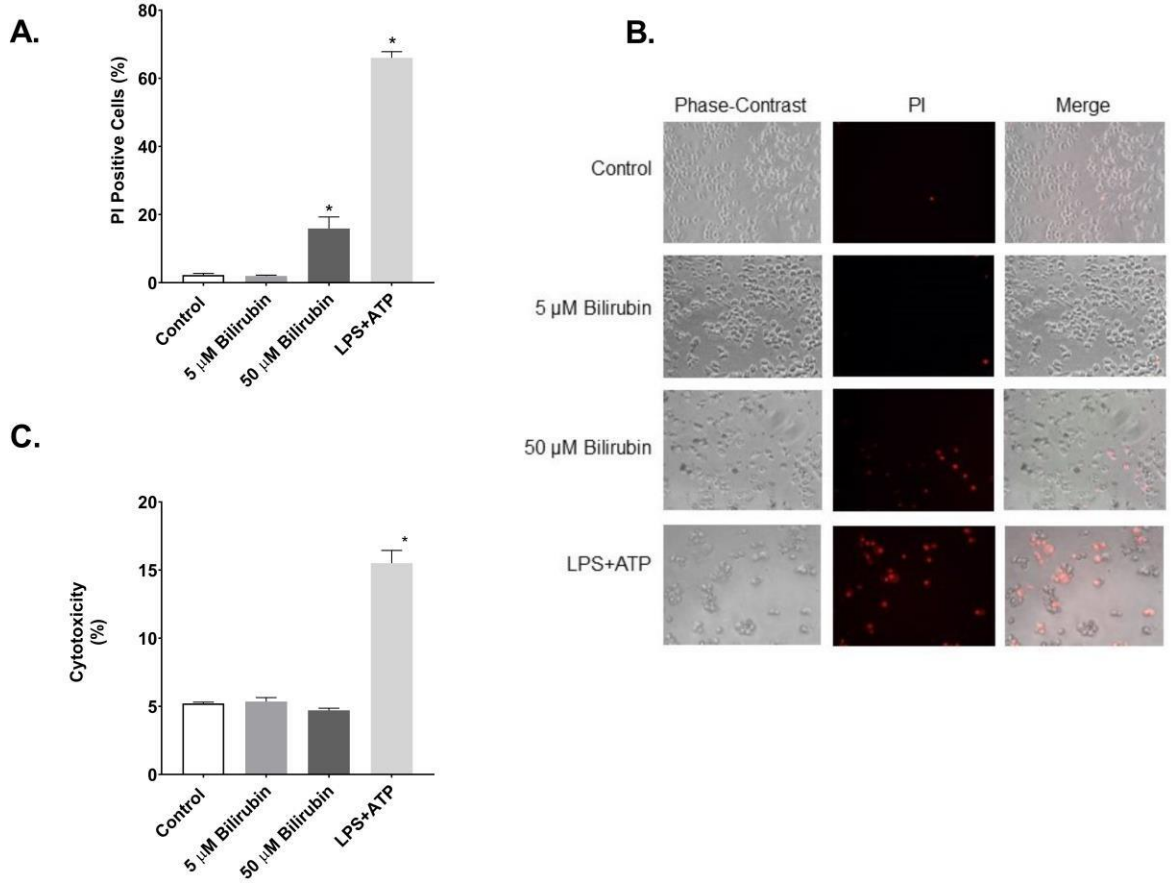

Figure 5: Effect of variable doses of bilirubin on cell toxicity and proptosis. Toxicity of variable concentrations of bilirubin was examined in order to find the optimal dose. (A, B) The amount of PI-positive cells increased when bilirubin at 50 µM caused pyroptotic cell death. (C) No result with LDH assay. LPS+ATP used as apositive control. The results are presented as mean ± S.E.M, n = 5. *p<0.05 and
**p<0.01 compared with untreated control.

4.4. Bilirubin promotes inflammasome parameters as detected by western blot analysis

To demonstrate inflammasome activation by bilirubin, western blot was performed using NLRP3, IL-1β and Caspase-1 antibodies after 50 µM bilirubintreatment for 6h in N9 microglial cells. Compared with control, 50 µM bilirubin was found to have an effect on inflammasome activation using both lysate and supernatant samples. Analysis of protein expression demonstrated that intracellular NLRP3 and secreted IL-1β protein level were significantly augmented due to bilirubin treatment. Because IL -1β release upon inflammasome activation depends on caspase-1 activation, caspase-1 protein level was determined. Bilirubin increased the secreted active caspase-1 (p20) protein level according to band intensities. In agreement with the results of the corresponding gene expression, hyperbilirubinemia can contribute the inflammasome activation in microglial cells **(Figure 6)**.

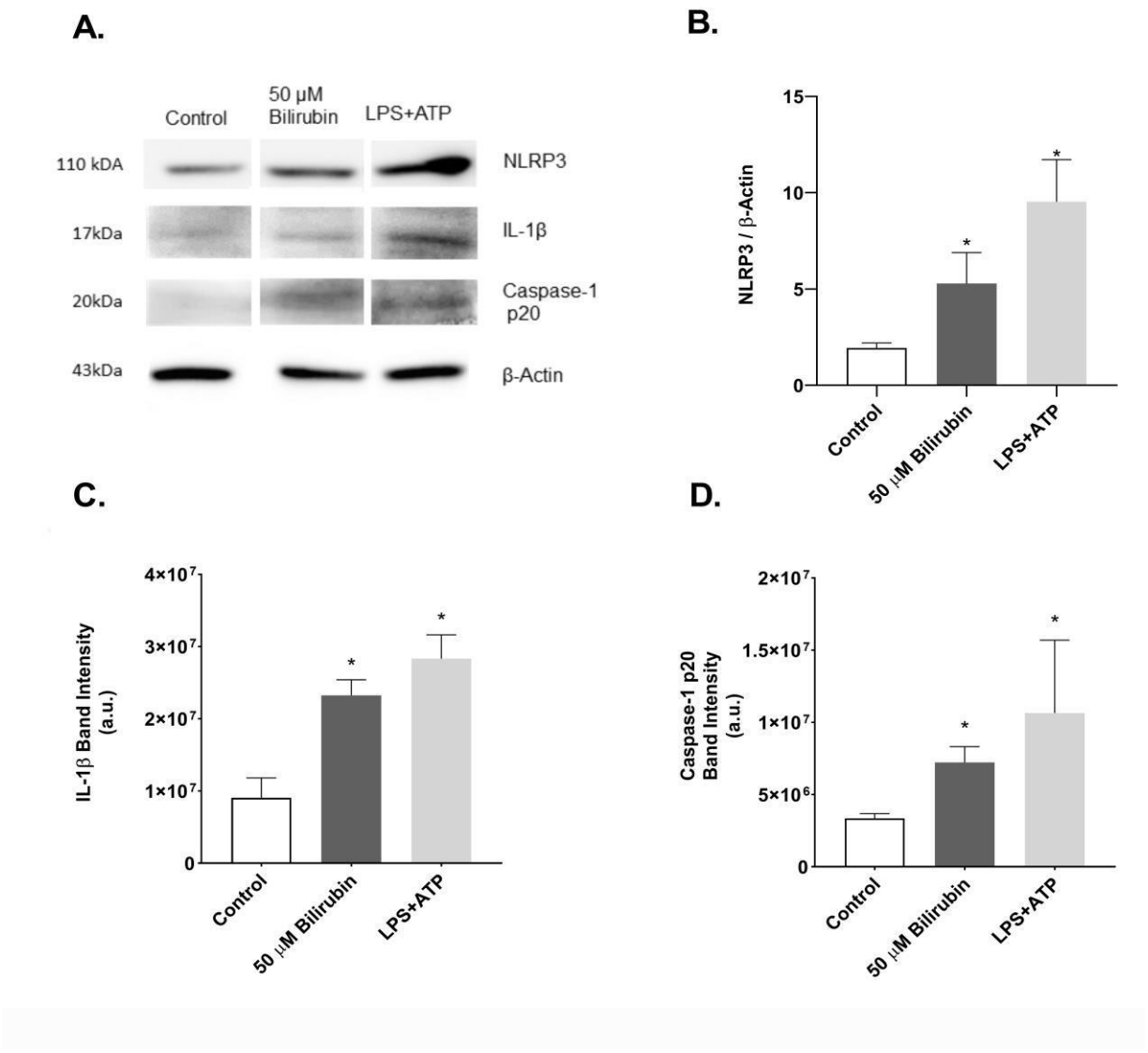

Figure 6: Effect of bilirubin on protein level of inflammasome parameters. (A) The protein levels of (B) NLRP3, (C) secreted IL-1β, and (D) cleaved caspase-1 (p20) were higher compared to the control. LPS+ATP used as a positive control. The results are presented as mean ± S.E.M, n = 5. *p<0.05 and **p<0.01 compared with untreated control.

4.5. Determination of bilirubin-activated inflammasome by *in vitro* knockdown method whether it is caused by NLRP3

After transfection and 50 µM bilirubin incubation, total RNA and supernatant proteins were obtained from N9 microglia cells treated with negative siRNA and NLRP3 siRNA to analyze the IL-1β and cleaved caspase-1 (p20) protein levels by western blot.

Firstly, transfection efficiency was detected by RT-PCR. NLRP3 mRNA expression change was found to be reduced by 37% in cells using NLRP3 siRNA. Thus, transfection and gene silencing were shown to be efficient. According to western blot results, In 50μM bilirubin-induced inflammasome, the fold change of IL-1β protein increased by 82% in negative siRNA, while increased by 20% in NLRP3 silenced cells. Cleaved caspase-1(p20) protein level increased by 82% in negative siRNA while decreased by -98% in NLRP3 siRNA transfected cells. Fold change in protein levels of IL -1β and caspase-1 show that the bilirubin effect was completely abolished in NLRP3- siRNA-transfected cells **(Figure 7).**

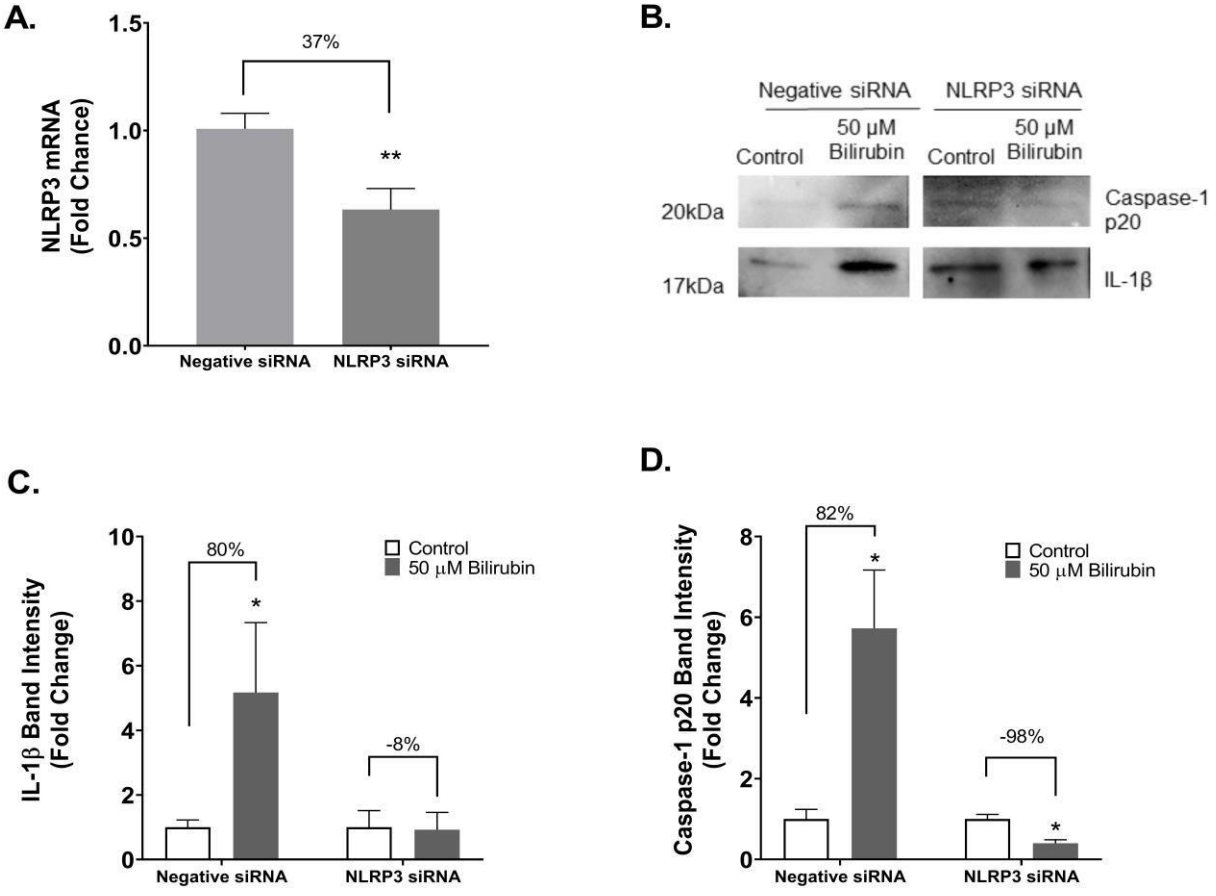

Figure 7: Elimination of NLRP3 decreases the release of inflammasome markers. (A) The transfection efficiency in control groups were detected by RT-PCR. (B, C, D) NLRP3 siRNA reduce the effect of bilirubin in terms of IL-1β and cleaved

caspase-1 (p20) protein levels. The results are presented as mean ± S.E.M, n = 5. *p<0.05 and **p<0.01 compared with untreated control.

4.6. Creation of an animal model of hyperbilirubinemia

Hyperbilirubinemia animal model was created by injection of bilirubin into cisterna magna punction. Three mice were used as a preliminary trial to check if the bilirubin colocalize the section of brain. Bilirubin was observed macroscopically at different levels of cross-sectional levels in obtained frozen sections from hyperbilirubinemia animal brains **(Figure 8)**

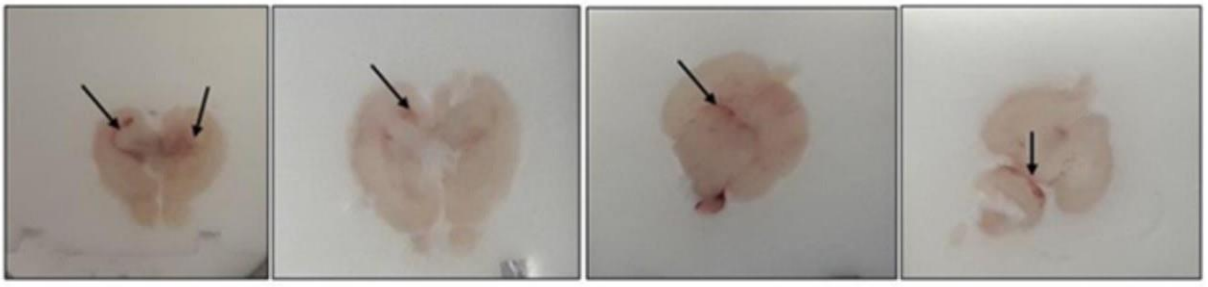

Figure 8: Experimental animal model of hyperbilirubinemia: After the bilirubinemia model was created, it was observed that there was bilirubin in the tissue at different levels of brain tissue sections. Black arrow: Showing bilirubin.

4.7. *In vivo* determination of the relationship of bilirubin with inflammasome by RT-PCR

To determine whether bilirubin caused an inflammasome in mouse brain *in vivo*, RT-PCR was performed with RNAs obtained from half brains extracted from animals treated with 10 µg and 20 µg bilirubin. Significant increases in IL-1β and NLRP3 mRNA expression change were observed in both groups **(Figure 9).**

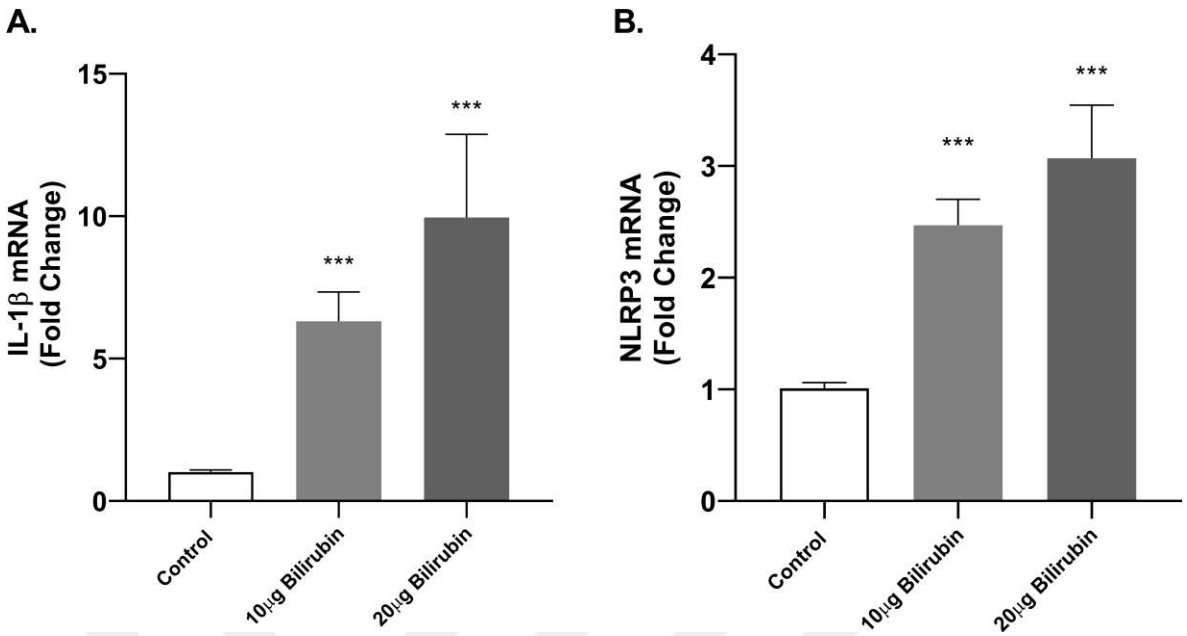

Figure 9: *In vivo* effects of bilirubin on the mRNA levels of the inflammasome indicator genes. (A) IL-1β (B) NLRP3. The results are presented as mean ± S.E.M, n = 5. *p<0.05, **p<0.01 and ***p<0.001 compared with untreated control.

4.8. Bilirubin promoted NLRP3-dependent inflammasome activation in mouse brain

For the sake of understanding the bilirubin-related inflammasome activation associated with NLRP3, 10 μg and 20 μg bilirubin received *Nlrp3* $^{-/-}$ and *Caspase-1*$^{-/-}$animals were used and band intensities were compared with wild-type animals.

Protein levels from half brains showed that both WT and Caspase-1-/- mice had higher NLRP3 protein levels after exposure to 10 μg and 20 μg bilirubin. The level of cleaved caspase-1 protein (p20) was also augmented in bilirubin-induced WT animals, but no secretion was observed in *Nlrp3*$^{-/-}$ brains. Furthermore, WT animals had higher levels of the IL-1β protein; however, in the brains of Nlrp3$^{-/-}$ and Caspase-1$^{-/-}$ mice, there were no bands to be seen. This result indicates that the absence of NLRP3 can avoid activation of caspase-1 caused by bilirubin. Similarly, knockout of NLRP3 and caspase-1 separately inhibited IL -1β secretion in bilirubin-injected mouse brains **(Figure 10)**.

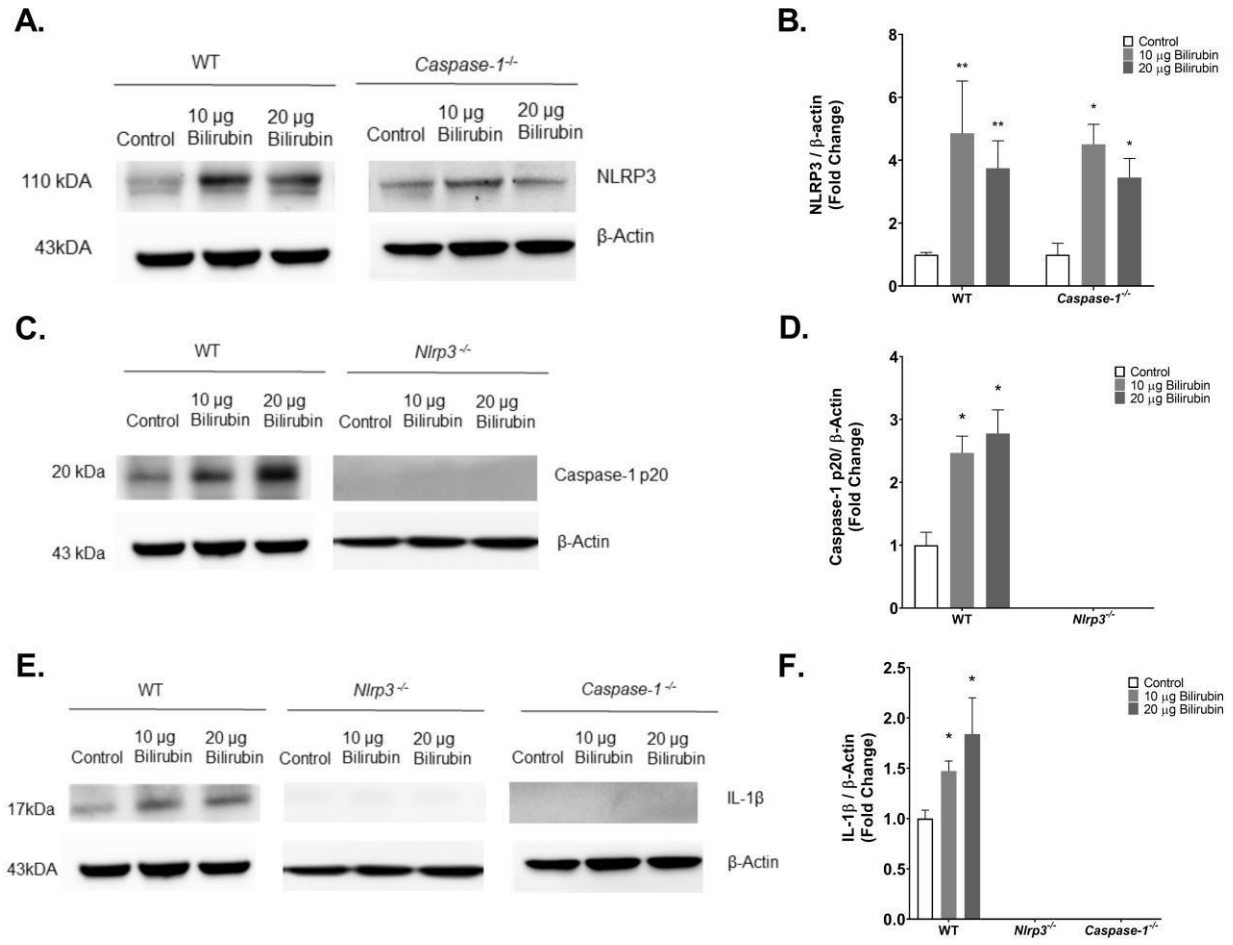

Figure 10: Bilirubin elevates the protein level of NLRP3 inflammasome parameters in mouse brains. (A, B) NLRP3 protein level; (C, D) cleaved caspase-1 (p20) and (E, F) secreted IL-1β protein level specified that inflammasome activation isrelated with NLRP3. The results are presented as mean ± S.E.M, n = 5. *p<0.05 and
**p<0.01 compared with untreated control.

5. DISCUSSION

Significant jaundice affects 20 million infants annually around the world, and hyperbilirubinemia is one of the main causes of neonatal deaths or long-term neurological impairments. Providing knowledge about BIND's mechanisms could be the first step toward lowering the disease burden. (Olusanya, Teeple et al. 2018). The

breakdown of nerve cells and mitochondrial membranes, the prevention of glutamate uptake, N-methyl-D-aspartic acid (NMDA)-mediated toxicity, and an increase in intracellular calcium have all been reported as side effects of bilirubin's cytotoxic effects in the central nervous system (Fernandes, Falcao et al. 2006). The present thesis proved that bilirubin activates the inflammasome and that NLRP3 is involved in this activation through *in vitro* and *in vivo* experiments.

Numerous in vivo simulations of the consequences of elevated bilirubin in animals have been proposed. The species was known as "Gunn rats" after Gunn's 1938 discovery of a novel mutation that caused jaundice. Gunn rat models were unable to help in the identification of the precise mechanisms underlying neonatal hyperbilirubinemia because hyperbilirubinemia peaks on a postnatal day 16 (Rice and Shapiro 2008). A mouse model of hyperbilirubinemia with the UDP glucuronosyltransferase 1 family (Ugt-1) gene knocked out was created by Nguyen and Fujiwara. But because the Ugt1 locus was disrupted, a lethal mutation was produced, and as a result, the newborn Ugt-1 null animals lost within two weeks of birth. This posed a serious obstacle to the investigation of bilirubin neurotoxicity (Nguyen, Bonzo et al. 2008). Therapeutic agents have been administered via cisterna magna puncture in some studies on central nervous system diseases. Using neonatal Sprague-Dawley rats and bilirubin injections into the cisterna magna, a new kernicterus model was developed in 2014. (Song, Hu et al. 2014). In this study, WT, Nlrp3$^{-/-,}$ and Caspase-1$^{-/-}$ animals received direct injections of bilirubin into the cisterna magna. To our knowledge, our team is the first to use this technique on mice. Because knock-out strains are more prevalent in mice than in rats, we hypothesize that this model will allow for the logical design of research studies aimed at determining the mechanisms at the molecular level that cause bilirubin neurotoxicity.

It has been claimed that nervous system glial cells exhibit variable degrees of sensitivity to bilirubin (Kumral, Genc et al. 2005, Brites 2011). It has been established that bilirubin negatively affects astrocyte cell viability, morphology, and mitochondrial function (Amit and Brenner 1993). Additionally, rat primary astrocytes were used to study the inflammatory effects of bilirubin. One of the studies' findings suggests that pro-inflammatory cytokines like TNF-, IL-6, and IL-1 can be released by 50 µM bilirubin

via the NF-κB and MAPK pathways. (Fernandes, Falcao et al. 2007). It was also demonstrated that inhibition of caspase-1 prevented the release of cytokines caused by bilirubin at a concentration of 50 µM, which caused pyroptosis in rat cortical astrocytes. (Feng, Li et al. 2018).

It has been established that microglia, which are resident macrophages and sensor cells in the central nervous system, are crucial for tissue repair and the control of inflammatory and immune responses. (Kreutzberg 1996). The presence of bilirubin pigment in the microglia was discovered by microscopic examination of kernicterus- covered sections of brain tissue (Gourley 1997). Although an increase in phagocytosis is most likely the microglia's initial response to bilirubin, it has been shown that an increase in apoptosis can occur after just 12 hours of incubation. (Silva, Vaz et al. 2010). According to research, higher bilirubin levels cause the microglia to become activated, exhibit inflammatory and phagocytic phenotypes, and release TNF-, IL-1, and IL-6 cytokines (Gordo, Falcao et al. 2006, Silva, Vaz et al. 2010). According to these studies, the pro-inflammatory status could generally reach its peak within 4 to 12 hours when there is a presence of 50 µM bilirubin. Bilirubin concentration was chosen based on published studies(Falcao, Fernandes et al. 2005, Gordo, Falcao et al. 2006, Silva, Vaz et al. 2010), and it was discovered that after 6 hours of incubation with 50 µM of bilirubin, N9 microglial cells developed both non-toxic and inflammatory effects.

Acute kidney injury or liver conditions are known to cause elevated bilirubin which is the main factor in neonatal jaundice. NLRP3 inflammasome activation typically represents a concomitant collateral effect in these conditions. For instance, it was discovered that elevated serum levels of bilirubin were significantly correlated with the expression of NLRP3, IL-1, IL-18, and procaspase-1 in cases of acute-on-chronic liver failure caused by the hepatitis B virus, bile cast nephropathy, and liver cirrhosis. (Ma, Zheng et al. 2018, Li and Jiang 2021, Shi, Wang et al. 2022). However, there are few studies describing the exact connection between bilirubin and NLRP3 inflammasome, particularly in the brain.

In this research, it was proposed that NLRP3 inflammasome activation is affected by bilirubin toxicity in the brain. According to results, incubating with 50 µM bilirubin

caused the N9 microglial cells to express more NLRP3 and IL-1β, which triggered theNLRP3 inflammasome priming stage. Additionally, it was showed that NLRP3, cleaved caspase-1 (p20), and cleaved IL-1β protein levels increased during the activation phase of NLRP3 inflammasome. It was clear that the levels of inflammasome parameters in vitro decreased when NLRP3 was silenced with a particular siRNA, which aided in understanding the role of NLRP3 in bilirubin related inflammasome activation. Li and Huang showed that caspase-1 and NLRP3 were activated throughoutthe rat model of kernicterus, and they hypothesized that VX-765's inhibition of caspase-1 could partially reverse the neurotoxicity caused by bilirubin (Li, Huang et al. 2021). This study examined the relationship between bilirubin toxicity and the inflammasomeusing NLRP3 and caspase-1 knockout mice. Because NLRP3 and caspase-1 are absent in mutant mouse brains, we came to the conclusion that the bilirubin effect couldnot be the same as in WT mouse brains. Increased levels of NLRP3, IL-β1, and caspase-1 proteins were found in WT animals, but only the NLRP3 protein level was noticed in Caspase-$1^{-/-}$ animals after bilirubin injection. In the activation step of inflammasome, the NLRP3 complex did not form because there was no IL-1β level inknockout animals however, NLRP3 was still present during the priming phase. These findings imply that one of the underlying mechanisms of bilirubin toxicity may involve the NLRP3 inflammasome. Additionally, according to Li and Huang, pyroptosis contributed to the pathogenesis of bilirubin neurotoxicity in kernicterus model rat brains. (Li, Huang et al. 2021). It was confirmed that bilirubin-induced pyroptosis in N9microglia with PI staining and caspase-1 activation in this study.

Numerous studies examining bilirubin neurotoxicity in new-borns have revealed that bilirubin primarily damages neurons. The primary targets of bilirubin toxicity have been identified as neurons due to bilirubin's tendency to attach to cell membranes, particularly myelin-rich membranes. (Watchko and Tiribelli 2013). *In vitro* studies showed that there was a reduction in neurite extension and dendritic and axonal branching. (Fernandes, Falcao et al. 2009), also decreased cell proliferation and an increase in apoptosis, (Falcao, Silva et al. 2007), commonly seen in neurons that havebeen exposed to bilirubin. (Fernandes, Falcao et al. 2009). It is well known that neuroinflammation frequently coexists with neurotoxicity. (Glass, Saijo et al. 2010).

Thus, neuroinflammation was used to clarify how bilirubin accumulated and caused neuronal cell death in Ugt1$^{-/-}$ mice. (Vodret, Bortolussi et al. 2017). Additionally, bilirubin-induced microglia produce high levels of nitric oxide as a result of glutamate uptake, which mediates inflammation-related neuronal cell death. (Silva, Vaz et al. 2012).

This study demonstrates a strong correlation between bilirubin-induced toxicity and activation of the microglial NLRP3 inflammasome. Researchers may have new opportunities to suggest innovative treatment plans for BIND if the mechanisms governing this association are further clarified.

6. CONCLUSIONS AND FUTURE DIRECTIONS

In this study, it was investigated whether bilirubin causes inflammasome activation in neonatal brain b *in vivo* and *in vitro* methods. *İn vitro* experiments, bilirubin application in the N9 microglia cell line was molecularly determined by ELISA, RT-PCR and western blot methods. In addition, NLRP3 role in inflammasome were confirmed by functional experiments with NLRP3 siRNA inhibition. *İn vivo* experiments, similarly increased doses of bilirubin have been shown to activate inflammasome activation in wild type mice and these results have been confirmed in NLPR3 $^{-/-}$ and Caspase-1 $^{-/-}$ hyperbilirubinemia mouse models.

For the future studies, the mechanisms of inflammasome caused by bilirubin and the regulation of inflammasome activation due to bilirubin was planned to investigate. For this aim, role of non-coding RNAs in this activation thought to be considered in other projects.

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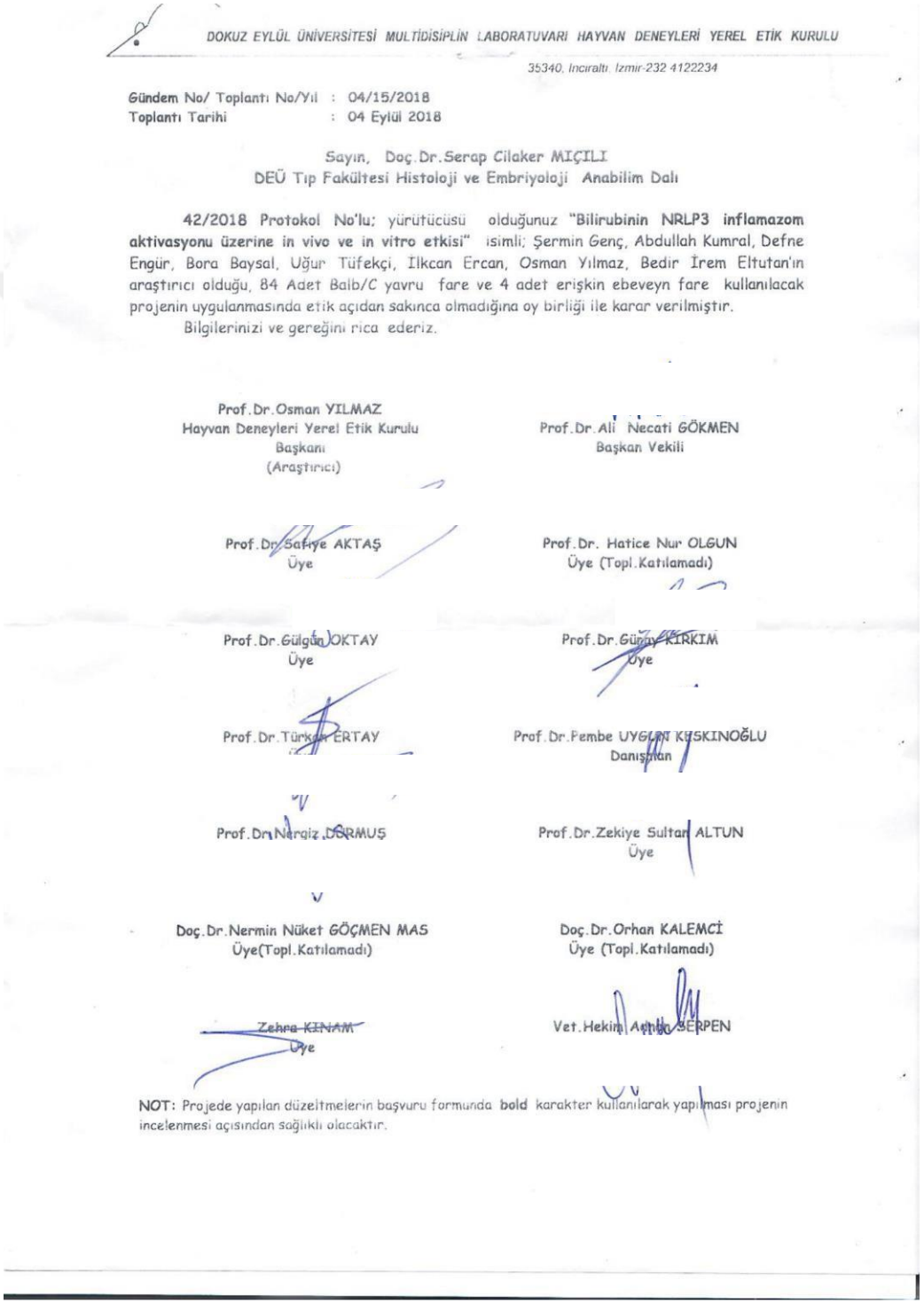
8. APPENDİX

8.1. Ethics Committee Report



DOKUZ EYLÜL ÜNİVERSİTESİ MULTİDİSİPLİN LABORATUVARI HAYVAN DENEYLERİ YEREL ETİK KURULU

35340, İnciraltı, İzmir-232 4122234

Gündem No/ Toplantı No/Yıl : 04/15/2018
Toplantı Tarihi : 04 Eylül 2018

Sayın, Doç.Dr.Serap Cilaker MIÇILI
DEÜ Tıp Fakültesi Histoloji ve Embriyoloji Anabilim Dalı

42/2018 Protokol No'lu; yürütücüsü olduğunuz **"Bilirubinin NRLP3 inflamazom aktivasyonu üzerine in vivo ve in vitro etkisi"** isimli; Şermin Genç, Abdullah Kumral, Defne Engür, Bora Baysal, Uğur Tüfekçi, İlkcan Ercan, Osman Yılmaz, Bedir İrem Eltutan'ın araştırıcı olduğu, 84 Adet Balb/C yavru fare ve 4 adet erişkin ebeveyn fare kullanılacak projenin uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.

Prof.Dr.Osman YILMAZ
Hayvan Deneyleri Yerel Etik Kurulu
Başkanı
(Araştırıcı)

Prof.Dr.Ali Necati GÖKMEN
Başkan Vekili

Prof.Dr.Safiye AKTAŞ
Üye

Prof.Dr. Hatice Nur OLGUN
Üye (Topl.Katılamadı)

Prof.Dr.Gülgün OKTAY
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Prof.Dr.Pembe UYGUN KÜSKINOĞLU
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Doç.Dr.Orhan KALEMCİ
Üye (Topl.Katılamadı)

Zehra KINAM
Üye

Vet.Hekim Aşkın SERPEN

NOT: Projede yapılan düzeltmelerin başvuru formunda **bold** karakter kullanılarak yapılması projenin incelenmesi açısından sağlıklı olacaktır.

İZMİR BİYOTIP VE GENOM MERKEZİ
HAYVAN DENEYLERİ YEREL ETİK KURULU (İBG-HADYEK)
KARARI

Toplantı Tarihi	: 15/01/2020	**Toplantı Günü**	: Çarşamba
Toplantı Sayısı	: 1	**Toplantı Saati**	: 15:30

Sayın Doç.Dr. Serap CİLAKER MIÇILI,

2020-001 Protokol No'lu; yürütücüsü olduğunuz "'Bilirubinin NRLP3 inflamazom aktivasyonu üzerine in vivo ve in vitro etkisi'" başlıklı projenin uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.

Doç. Dr. Güneş ÖZHAN BAYKAN **Başkan**	**Vet. Hek. Kerem ESMEN** **Başkan Yardımcısı**
Prof. Dr. H. Alper BAĞRIYANIK **Üye**	**Prof. Dr. Ensari GÜNELİ** **Üye**
Doç. Dr. Kasım DİRİL **Üye**	**Dr. Öğr. Üyesi Ayşe Banu DEMİR** **Üye**
Dr. Gülçin ÇAKAN AKDOĞAN **Üye** (Katılmadı)	**Vet. Hek. Umur KELEŞ** **Üye**
H. Muammer KARAGÖZ **Üye**	

8.2. Curriculum Vitae



İLKCAN ERCAN ORHAN

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Contact Information

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Telephone	(
Email	i
Web Page	

Education Information

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Cumulative Grade Point Average: 2.69 / 4.0

Experience Information

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01 June 2016 - 01 September 2017 (1 Year 4 Month) (Tam Zamanlı)
DİĞER, İNTERLAB LABORATUAR ÜRÜNLERİ SAN. VE TİC. A.Ş.

Scientific Technological Activity Fields

Keywords

İnflamazom
Mikroglia
Nöroenflamasyon

R&D Competency

Articles

B. I. ELTUTAN, C. KİSER, I. ERCAN, K. U. TUFEKCİ, D. ENGUR, S. GENC & A. KUMRAL, In vitro effects of H2O2 on neural stem cell differentiation, IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY-ANIMAL, 2022, 1071-2690, 58, 9, 810-816.

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Conference Papers

Ç. KİSER, C. P. GÖNÜL, İ. ERCAN, D. ENGÜR, A. KUMRAL & Ş. GENÇ, The Effect of M1 and M2 polarized microglia-derived exosomes on neural stem cell differentition, Poster Sunumu, FENS 2022, 09 July 2022, 13 July 2022.

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İ. , Microglial phenotypes are determined by variable intracellular hydrogen peroxide concentrations, Poster Sunumu, XIV European Meeting on Glial Cells in Health and Disease (Glia 2019), 10 July 2019, 13 July 2019.

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TÜBİTAK Scholarships and Fundings

Project Info	212T186, Sistemik Mantar İlaçlarına Karşı Gelişen İlaç Dirençlilik Mekanizmalarının Belirlenmesi, Uluslararası, Burslu, Sonuçlandı, ARDEB, KBAG - Kimya Biyoloji Araştırma Destek Grubu, Project Participation/Leave Dates: 10/1/13 - 7/15/15, Project Start/End Dates: 7/15/13 - 7/15/15. 2175584, Alzheimer Hastalığının Tanısında ve Yayılımında Nöron Kökenli Ekzozomlar, 1001 - Araştırma, Burslu, Sonuçlandı, ARDEB, SBAG - Sağlık Bilimleri Araştırma Destek Grubu, Project Participation/Leave Dates: 7/23/18 - 6/1/19, Project Start/End Dates: 6/1/18 - 6/1/20. 2185530, Bilirubinin NLRP3 Inflamazom Aktivasyonu Üzerine In Vivo ve In Vitro Etkisi, 1001 - Araştırma, BİDEB Burslusu, Sonuçlandı, ARDEB, SBAG - Sağlık Bilimleri Araştırma Destek Grubu, Project Participation/Leave Dates: 2/1/21 - 9/1/21, Project Start/End Dates: 9/1/19 - 9/1/21. 2185527, Polarize Mikroglia Kökenli Ekzozomal mikroRNA'ların Nöral Kök Hücre Farklılaşması Üzerine Etkilerinin Değerlendirilmesi, 1001 - Araştırma, BİDEB Burslusu, Yürürlükte, ARDEB, SBAG - Sağlık Bilimleri Araştırma Destek Grubu, Project Participation/Leave Dates: 10/22/21 - 12/15/22, Project Start/End Dates: 6/15/19 - 12/15/22. 122Z771, Depresif Bozuklukta Mitokondriyal Dna Değişiklikleri, 1001 - Araştırma, Burslu, Yürürlükte, ARDEB, KBAG - Kimya Biyoloji Araştırma Destek Grubu, Project Participation/Leave Dates: 1/16/23 - 11/15/25, Project Start/End Dates: 11/15/22 - 11/15/25.
BIDEB Supports	İLKCAN ERCAN ORHAN, Araştırma Burs ve Destekleri Müdürlüğü, 2218-Yurt İçi Doktora Sonrası Araştırma Burs Programı, Burs Almaya Hak Kazandı, 2021 - 2. İLKCAN ERCAN ORHAN, Etkinlik Destekleri ve Eğitim Bursları Müdürlüğü, 2250-Lisansüstü Bursları Performans Programı, Destek Sona Erdi, 2022 - 1. İLKCAN ERCAN ORHAN, Etkinlik Destekleri ve Eğitim Bursları Müdürlüğü, 2211-A Genel Yurt İçi Doktora Burs Programı, Destekleniyor, 2019 - 2, 6/1/19 - 5/31/23.

Number of Panelist/Observer/Reporter Jobs

Refereeing/Panelist/Outer Consultant Count	ARDEB/BİDEB 0	TEYDEB 0	Total 0
Number of Observer/Consultant Jobs	ARDEB/BİDEB 0	TEYDEB 0	Total 0
Number of Reporter Jobs	ARDEB/BİDEB 0	TEYDEB 0	Total 0

8.3. Publications from the thesis

Ercan I, Cilaker Micili S, Soy S, Engur D, Tufekci KU, Kumral A, Genc S. Bilirubin induces microglial NLRP3 inflammasome activation in vitro and in vivo. Mol Cell Neurosci. 2023 Mar 23;125:103850. doi: 10.1016/j.mcn.2023.103850. Epub ahead of print. PMID: 36965549.

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Bilirubin induces microglial NLRP3 inflammasome activation *in vitro* and *in vivo*

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ARTICLEINFO

Keywords:
Bilirubin-induced neurological dysfunction (BIND)
Kernicterus
NLRP3 inflammasome
Microglia
Neuroinflammation

ABSTRACT

Despite current advancements in neonatal care, hyperbilirubinemia resulting in bilirubin-induced neurological dysfunction (BIND) continues to be one of the major reasons of mortality or lifelong disability. Although the exact mechanisms underlying brain injury upon bilirubin exposure remains unelucidated, inflammation is considered to be one of the major contributors to BIND. This study investigates the role of the NLRP3 inflammasome in bilirubin-induced injury using *in vitro* and *in vivo* models. We successfully demonstrated that the upregulation of NLRP3 expression is significantly associated with the release of active caspase-1 and IL-1β in N9 microglial cells exposed to bilirubin. Functional *in vitro* experiments with NLRP3 siRNA confirms that bilirubin-induced inflammasome activation and cell death are mediated by the NLRP3 inflammasome. Following injection of bilirubin into the *cisterna magna* of a neonatal mouse, activation of the NLRP3 inflammasome and microglia were determined by double staining with Iba1-NLRP3 and Iba1-Caspase-1. Upon injection of bilirubin into the *cisterna magna*, neuronal loss was significantly higher in the wild-type mouse compared to *Nlrp3*$^{-/-}$ and *Caspase-1*$^{-/-}$ strains. Collectively, these data indicate that NLRP3 inflammasome has a crucial role in microglial activation and bilirubin-induced neuronal damage.

1. Introduction

Hyperbilirubinemia is a commonly encountered condition in neonatology, and increased levels of unconjugated bilirubin can evenly pass through the immature blood-brain barrier, thereby leading to adverse consequences. During the neonatal period, bilirubin can damage susceptible regions of the developing central nervous system, *i.e.*, basal ganglia, cerebellum, and brainstem, causing permanent neurological deficits termed as Bilirubin-Induced Neurological Dysfunction (BIND) syndrome. As a result of the advances in neonatal care, there has been a marked decrease in the incidence reporting of BIND in western countries; however, it still represents a major cause of mortality or lifelong disability in the developing world (Kang et al., 2020). Currently, BIND can be prevented by the early identification of infants with risk factors for hyperbilirubinemia, close neonatal screening before and after hospital discharge, along with the timely application of phototherapy and exchange transfusion in the indicated cases; nevertheless, a definitive treatment does not exist for this chronic condition (Karimzadeh et al., 2020).

The underlying mechanisms of bilirubin neurotoxicity and BIND have not been fully elucidated; however, inflammation has been proposed as a significant contributor to those syndromes (Vodret et al., 2017; Vodret et al., 2018). As the critical mediators of inflammation, inflammasomes, are multiprotein complexes that act as intracellular sensors that detect and respond to several stimuli, such as infectious agents, certain toxins, or environmental agents. Upon activation, these

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cytosolic proteins form oligomers, leading to the initiation and propagation of inflammatory responses (Pandey et al., 2021). Nod-like receptor (NLR) subset inflammasomes constitute a family of pattern recognition receptors, and NLRP3 is a well-characterized prototype member of this family. The inflammasome complex consists of a sensor cytosolic NLRP3, an adapter "apoptosis-associated speck-like protein containing CARD" (ASC), and an effector pro-caspase-1 (Jo et al., 2016). Once the assembly of the NLRP3 inflammasome is triggered, it results in the activation of caspase-1 and secretion of pro-inflammatory cytokines, cellular damage, and pyroptotic cell death (Liston and Masters, 2017). It is well known that conditions involving inflammasome activation or dysregulation contribute to the pathogenesis of various disorders, including infections, autoimmune and neurodegenerative diseases (Jo et al., 2016).

Regarding the neonatal central nervous system, NLRP3 inflammasome activation has been linked to hypoxic and hyperoxic brain injury in newborns (Ystgaard et al., 2019; Micili et al., 2020); however, its role in BIND has not been studied in detail. We propose that the NLRP3 inflammasome could have a role in the pathogenesis of bilirubin neurotoxicity and long-term disabilities. To test our hypothesis, we first used an *in vitro* cell culture model and investigated the activation of the NLRP3 inflammasome following bilirubin exposure. We also carried out functional experiments through NLRP3 knockdown by siRNA transfection. Then, we generated a mouse model of hyperbilirubinemia *via* injection of bilirubin into the *cisterna magna* and evaluated the effects of hyperbilirubinemia in wild-type (WT), NLRP3 knock-out and Caspase-1 knock-out (*Nlrp3*$^{-/-}$ and *Caspase-1*$^{-/-}$) mice.

2. Materials and methods

2.1. Cell culture and treatment

Cell lines of murine microglia N9 were obtained from Paola Ricciardi-Castagnoli (Cellular Pharmacology Center, Milan, Italy) (Righi et al., 1989). Cells were cultured in RPMI1640 medium containing 2 mM L-Glutamine, 10 % FBS (Gibco, Thermo Fisher Scientific, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37 °C in a humid incubator with a 5 % CO2. Cells from passages 20 to 40 were treated with 50 μM bilirubin with or without 1 μg/ml Lipopolysaccharide (LPS) (Invivogen, France) for 6 h. A fresh bilirubin solution containing 0.1 NaOH was prepared immediately before each use. 0.1 M HCl was used to bring the pH of bilirubin in the growth media to 7.4. All the experiments with bilirubin were carried out under light protection.

2.2. ELISA

N9 microglial cells were treated with 50 μM bilirubin for 6 h, and the supernatant from the culture medium was collected and IL-1β protein levels were analyzed with an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.3. Propidium iodide (PI) staining

N9 microglial cells were seeded into a 96-well plate with the density of 10^4 cells/well, and the cells were treated by 50 μM bilirubin. After 6 h, 50 μg/ml PI dye (Thermo Fisher Scientific, Waltham, MA, USA) was added to each well, and images were captured under fluorescent microscopy. ImageJ software was used to quantify the total and dead cells.

2.4. Quantitative PCR (qPCR)

The Nucleospin RNA isolation kit (Macherey-Nagel, Germany) was used to extract total RNA from cells and animal tissues. The concentration of each RNA sample was measured using a NanoDrop spectrophotometer. Reverse transcription reaction was carried out with the High-Capacity cDNA Reverse Transcription Kit (ThermoScientific, USA). After obtaining cDNAs, mRNA expression levels were measured with GoTaq® qPCR Master Mix (Promega; USA) by using the ABI 7500 fast quantitative PCR device. The relative gene expression changes were normalized with GAPDH and quantified with the $2^{-\Delta\Delta Ct}$ method using Ct values (Livak and Schmittgen, 2001). The list of the primers is given in Table 1.

Table 1
The primer list and sequences (5′–3′) used in the qPCR reactions.

NLRP3	Forward	TGCCTGTTCTTCCAGACTGGTGA
	Reverse	CACAGCACCCTCATGCCCGG
IL-1β	Forward	CACAGCAGCACATCAACAAG
	Reverse	GTGCTCATGTCCTCATCCTG
GAPDH	Forward	ACCACAGTCCATGCCATCAC
	Reverse	TCCACCCTGTTGCTGTA

Table 2
Primary and secondary antibody information used in western blot analysis.

Antibody	Provider	Catalog number
Anti-NLRP3	Adipogen	Ag-20b-0014-c100
Anti-Iba1	Abcam	Ab178847
Anti-Caspase-1	Adipogen	Ag-20b-0042-c100
Anti-Neurofilament	Cell Signaling	2837S
Alexa Fluor® 488	Invitrogen	A11001
Alexa Fluor® 594	Invitrogen	A21125
Anti-IL-1β	Abcam	ab9722
Anti-Beta Actin	Abcam	ab8227
Anti-rabbit IgG	Cell signaling	7074S

2.5. Western blot

Following bilirubin treatment, protein samples from N9 microglial cells and brain tissues were extracted using the RIPA lysis buffer, including the protease and phosphatase inhibitor (Thermo Scientific, USA). Brain tissues were homogenized, and supernatant proteins were obtained after lyophilization. Protein concentrations were quantified with the BCA Assay kit (Takara Bio; Japan), and equal amounts of proteins were separated through 10 % SDS-PAGE gel electrophoresis. After transfer of the gels onto a polyvinylidene difluoride (PVDF) membrane, the membranes were blocked with skim milk or BSA according to antibody specificity to prevent nonspecific binding and probed with primary antibodies (Table 2) overnight at 4 °C followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Protein band signals were detected using Supersignal West Dura ECL reagent (ThermoScientific, USA) with the ChemiDoc Imaging System (Bio-Rad, California, USA). Band densities were scanned and analyzed using the Image Studio Lite (Version 5.2) software and Image Lab software (Version 6.1). The bands derived from the cell lysates were adjusted to the loading reference, β-actin. Results from analyses of secreted proteins were presented as ratios to corresponding control samples.

2.6. Transfection

50 nM NLRP3 siRNA (Dharmacon, Mouse Nlrp3 SMARTpool ON-TARGET siRNA, L-053455-00-0005) and negative control siRNA were transfected to N9 microglia cells by using siRNA Dharmafect transfection reagent (T-2001-03, Dharmacon, USA) in antibiotic-free complete RPMI 1640 medium according to manufacturer's instructions (Tufekci et al., 2021). 48 h after transfection, the cells underwent a 6 h incubation with bilirubin. For RT-PCR and western blot studies, the cells and supernatant were then gathered.

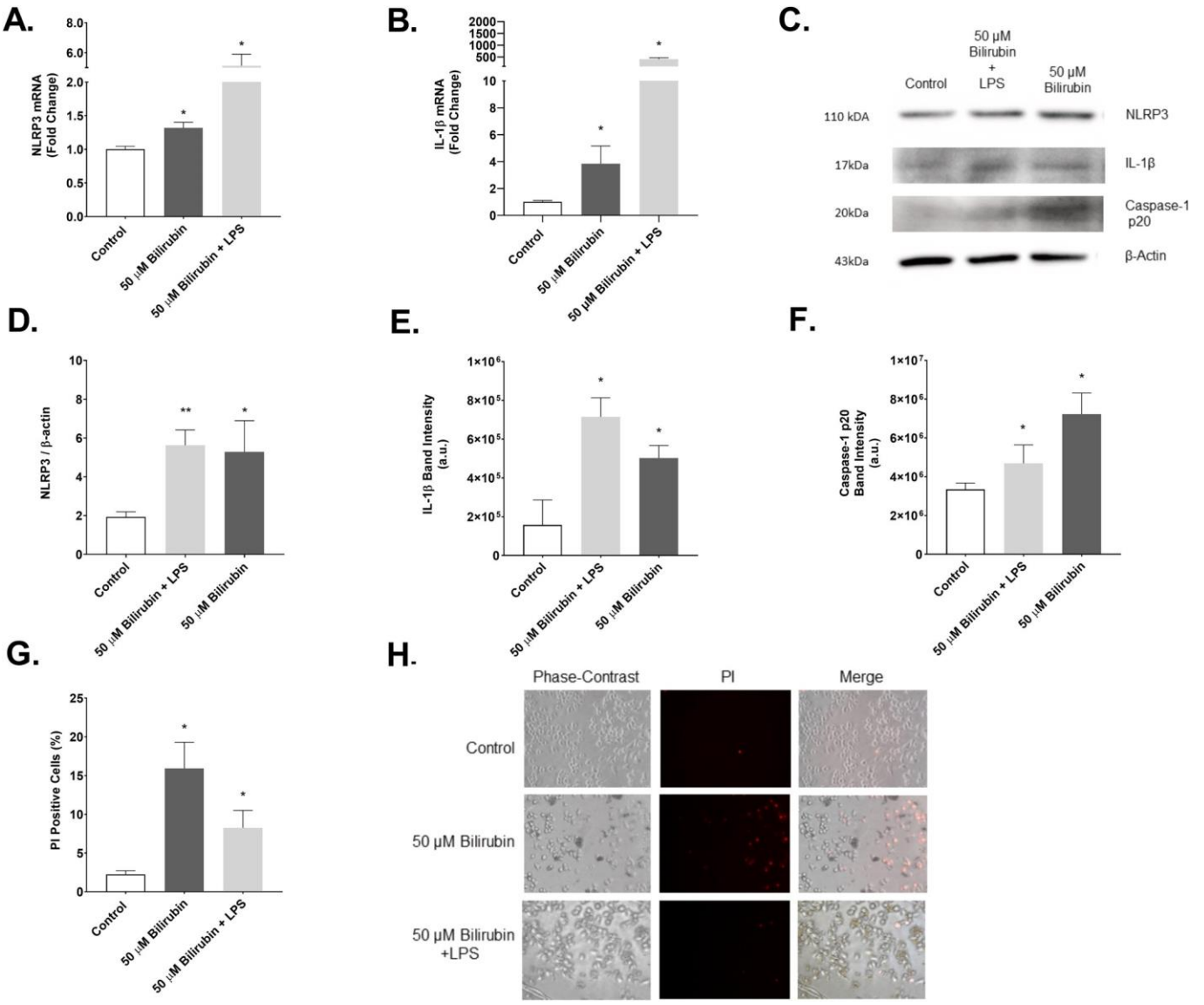

Fig. 1. Bilirubin promotes inflammasome activation and pyroptosis *in vitro*. N9 microglial cells were incubated either in the presence of 50 μM bilirubin or 50 μM bilirubin+LPS (1 μg/ml) or absence (control) for 6 h. (A, B) mRNA levels of IL-1β and NLRP3 were increased with 50 μM bilirubin. (C) The protein levels of (D) NLRP3, (E) secreted IL-1β, and (F) cleaved caspase-1 (p20) were higher compared to the control. (G, H) 50 μM bilirubin induced pyroptotic cell death and increased the number of PI-positive cells. The results are presented as mean ± S.E.M, n = 5. *p < 0.05 and **p < 0.01 compared with untreated control.

2.7. Animals and model establishment

All animal studies were performed according to the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of the Dokuz Eylul University School of Medicine for WT mice (Protocol number: 42/2018) and from Izmir International Biomedicine and Genome Institute Local Ethics Committee for knock-out animals (Protocol number: 01/2020). For the WT studies, C57BL/6 mice were used. NLRP3 knock-out (*Nlrp3*$^{-/-}$, B6.129S6-NLRP3 < TM1BHK>/J HOM) and Caspase-1 knock-out (*Caspase-1*$^{-/-}$, B6N·129S2-CASP1 < TM1FLV>/J HOM) mice in the C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, ME). At least five animals were utilized for each experimental condition. All animals were maintained in the vivarium under controlled conditions (23 ± 2 °C; 12 h light/dark periods) with access to food and water *ad libitum*.

Bilirubin (B4126, Sigma-Aldrich, USA) was dissolved in 0.5 M NaOH solution and diluted in ddH_2O to 10 mg/ml, and the pH was adjusted to 8.5 with HCl (0.5 M) under light protection (Song et al., 2014). Song et al. demonstrated that a 10 g/g (bodyweight) dose results in characteristic neurological manifestations as well as a better survival rate (Song et al., 2014). Therefore, we applied both 10 μg/g (bodyweight) and 20 μg/g (bodyweight) doses. On postnatal (PN) day 5, C57BL/6 mice pups from the litter were randomly divided into three groups: Control (n = 14), 10 μg/g bilirubin (n = 14), 20 μg/g bilirubin (n = 14). Bilirubin or dH2O was injected into the cisterna magna using a micro syringe (Song et al., 2014). Diethyl ether was used to anesthetize the pups. An equal volume of cerebrospinal fluid was obtained before application of bilirubin. The control group received an injection with normal saline. The experiment was finalized 24 h after injections, and animals were sacrificed by cervical dislocation. Brain samples were quickly fixed in 4 % PFA solution and dehydrated in 10 % sucrose solution for the histological examination. Tissues were embedded in OCT (Tissue-Tek) and serially sectioned with a cryostat. For the molecular examinations, brain tissues were kept at —80 °C.

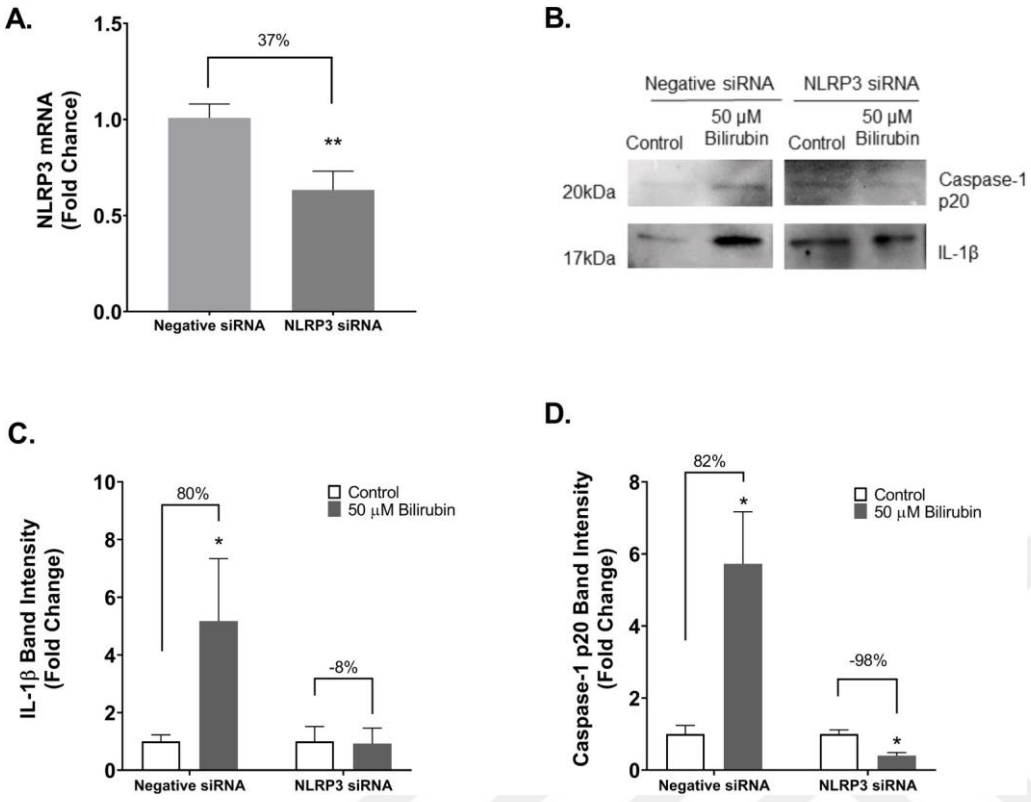

Fig. 2. NLRP3 elimination reduces the secretion of inflammasome markers *in vitro.* N9 cells were transfected with negative or NLRP3 siRNA for 48 h and then incubated with 50 μM bilirubin or control for 6 h. (A) The efficiency of transfection in control groups was determined by RT-PCR. (B, C, D) NLRP3 siRNA lowered the effect of bilirubin in terms of IL-1β and cleaved caspase-1 (p20) protein levels. The results are presented as mean ± S.E.M, n = 5. *p < 0.05 and **p < 0.01 compared with untreated control.

2.8. Histological examination

Immunohistochemistry was performed on 8 μm-thick prefrontal cortex (PC) (plate 8,9) and hippocampal sections (CA1) (plate 22, 23 according to Paxinos). First, tissues were fixed on slide glass air and dried for 20 min at room temperature (Micili et al., 2019). Next, slides were held in cold acetone for 7 min for permeabilization. Then, 4 % goat serum was administered for 1 h at room temperature to block nonspecific binding. After washing with 1× TBS containing 0.1 % Tween-20, the slides were incubated with the primary antibodies at 4 °C overnight (Table 2). After incubating tissue slides with fluorescent conjugated secondary antibodies for 1 h at room temperature, slides were washed three times with 1× PBS and then rinsed with dH_2O, and the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Images were visualized using a fluorescent microscope (Olympus BX-61, Japan) and analyzed with Image-J software (National Institutes of Health, USA) (Arioz et al., 2019).

At least five animals were used for immunohistochemical studies. Nine equal sized regions of interest with from each animal were examined. The average intensity was calculated and given as an arbitrary unit after adjustment for standard background. For co-localization analysis, immunofluorescent images were taken and the JACoP ImageJ plug-in was used to calculate Manders' coefficient (Zarand, 1981). Examiners worked blind to avoid experimental bias.

2.9. Statistical analysis

All data analyses were performed with GraphPad Prism 8.0 (Graphpad Software Inc., CA, USA). Shapiro–Wilk test was applied for the normality test. Since the data was not normally distributed, the Mann-Whitney *U* test was used to compare two independent groups, and the Kruskal-Wallis test was used to compare three or more groups. A pairwise comparison test based on Dunn's theorem was applied when a difference was detected in the Kruskal-Wallis test. The values were given as mean ± standard error of the mean (SEM). Analyses with a p-value <0.05 ($p < 0.05$) were considered statistically significant.

3. Results

3.1. Bilirubin induced inflammasome activation and pyroptotic cell death in N9 microglial cells

To investigate the effect of bilirubin on inflammasome activation, we treated N9 microglial cells with or without 50 μM bilirubin, and 50 μM bilirubin together with 1 μg/ml LPS for 6 h. Bilirubin+LPS group was added to detect a dual effect if any. We examined the mRNA levels to determine how bilirubin affects the transcription of IL-1β and NLRP3. According to the RT-PCR results, IL-1β and NLRP3 mRNA levels were significantly upregulated with 50 μM bilirubin treatment (Fig. 1A, B). In addition, protein expression analysis revealed that NLRP3, the mature form of IL-1β, and active caspase-1 protein levels were significantly elevated following bilirubin treatment (Fig. 1C–F). Given that NLRP3 activation triggers pyroptotic cell death, bilirubin-induced pyroptotic cell death was assessed by PI staining. Our results revealed that after 50 M bilirubin treatment, 16 % of the cells were PI-positive, indicating that bilirubin treatment induces pyroptotic death in microglial cells (Fig. 1G, H).

3.2. Bilirubin-mediated inflammasome activation is related to NLRP3 in N9 microglial cells

To examine whether inflammasome activation depends on NLRP3, inflammasome components in mRNA and protein levels were assessed in NLRP3 knockdown cells. A 37 % decrease in the NLRP3 mRNA expression (Fig. 2A) led to a reduction in the effect of bilirubin on the IL-1β and caspase-1 levels(Fig. 2B). Fold-change in the protein level of IL-1β and caspase-1 indicate that the bilirubin effect was utterly reversed in NLRP3 siRNA transfected cells (Fig. 2C–D).

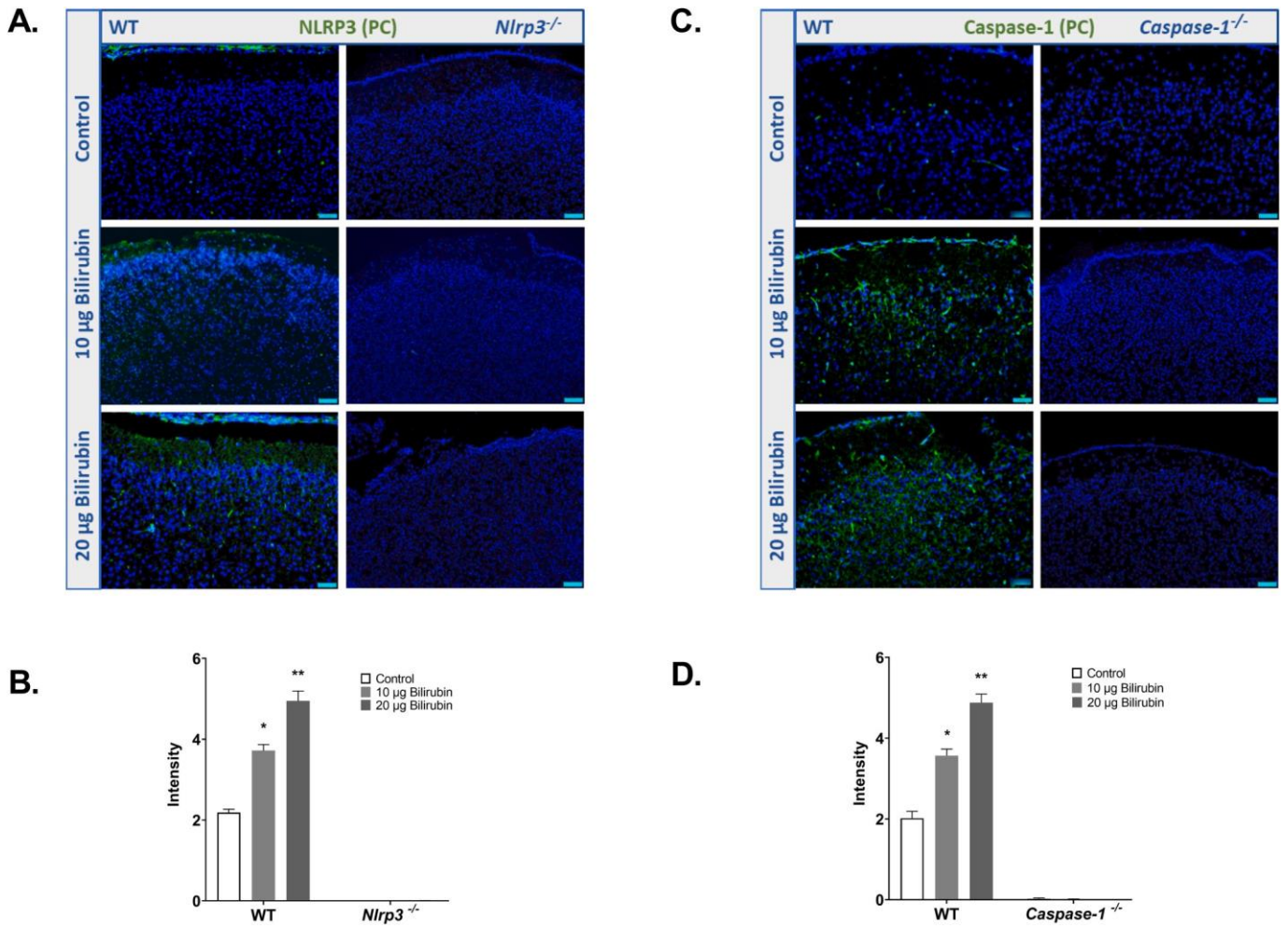

Fig. 3. NLRP3 and Caspase-1 immunofluorescence stain in mouse brain. Brain sections were stained with NLRP3 and Caspase-1 (green), and nuclei were stained with DAPI (blue). NLRP3 signaling was activated in the PC regions of mice brains following bilirubin injection. (A, B) In WT mice, NLRP3-positive cells were increased both in 10 ㎍g and 20 ㎍g bilirubin groups compared to the control, while no staining was observed in the *Nlrp3*$^{-/-}$ group. (C, D) Similarly, the number of Caspase-1 positive cells were increased with bilirubin treatment in WT mice, while no staining was observed in the *Caspase-1*$^{-/-}$ mice. Data are presented as mean ± S.E.M, n = 5. *p < 0.05 and **p < 0.01 compared to control group. Magnification: 10×.

3.3. Bilirubin promoted NLRP3-dependent inflammasome activation in mouse brain

In order to understand the effects of bilirubin on inflammasome activation, the NLRP3 and Caspase-1 levels in brain tissue were determined by immunofluorescence staining. In the WT animals, the NLRP3 and caspase-1 immunoreactive cells in the bilirubin groups were significantly increased compared to the control. However, neither NLRP3 activation in *Nlrp3*$^{-/-}$ animals nor Caspase-1 activation in *Caspase-1*$^{-/-}$ animals were observed (Fig. 3 and Supplementary Fig. 1).

We further confirmed our immunofluorescence results with immunoblotting analysis. Western blot band intensities revealed that 10 ㎍g and 20 ㎍g of bilirubin increased the NLRP3 protein levels in both WT and *Caspase-1* $^{-/-}$ mice (Fig. 4A, B). The cleaved Caspase-1 (p20) protein level was also increased by bilirubin treatment in WT animals, but not in *Nlrp3*$^{-/-}$ mice (Fig. 4C, D). Likewise, bilirubin treatment resulted elevation of IL-1β protein levels in WT animals, but not in *Nlrp3*$^{-/-}$ and *Caspase-1* $^{-/-}$ mice (Fig. 4E, F). These findings reveal that the absence of NLRP3 could prevent bilirubin-induced Caspase-1 activation. Knocking-out NLRP3 or caspase-1 both inhibited IL-1β production in the mouse model of hyperbilirubinemia.

3.4. Bilirubin promotes microglial activation

To investigate the effect of bilirubin on inflammasome activation in microglia, double immunofluorescence staining was performed in the PC region of WT mice. Iba1 was used as a specific marker for microglia. NLRP3 expression, as well as Iba1 level, were increased compared to the control (Fig. 5A). Furthermore, the co-localization study revealed that bilirubin-treated mice had higher levels of Iba1-NLRP3 co-localization. **(**Fig. 5B**)**.

3.5. Bilirubin causes neurofilament loss

Neurofilament (NF) immunofluorescence staining was performed in WT and knock-out strains to determine whether bilirubin causes neuronal damage. NF was used as a marker of neuronal intensity. In the WT mice, a significant decrease in NF staining in the PC1 region was observed with escalating doses (10 ㎍g and 20 ㎍g) of bilirubin compared to the control (Fig. 6A). With escalating doses of bilirubin, a substantial reduction in NF staining was also observed in the *Nlrp3*$^{-/-}$ and *Caspase-1*$^{-/-}$ mice (Fig. 6B, C), though this reduction was less pronounced than it was in the WT animals. In knock-out mice, there was a substantially reduced proportional ratio of neuronal loss between the control and bilirubin-treated groups. (Fig. 6D). The same results were observed in the CA1 regions of WT and knock-out animals (Supplementary Fig. 2).

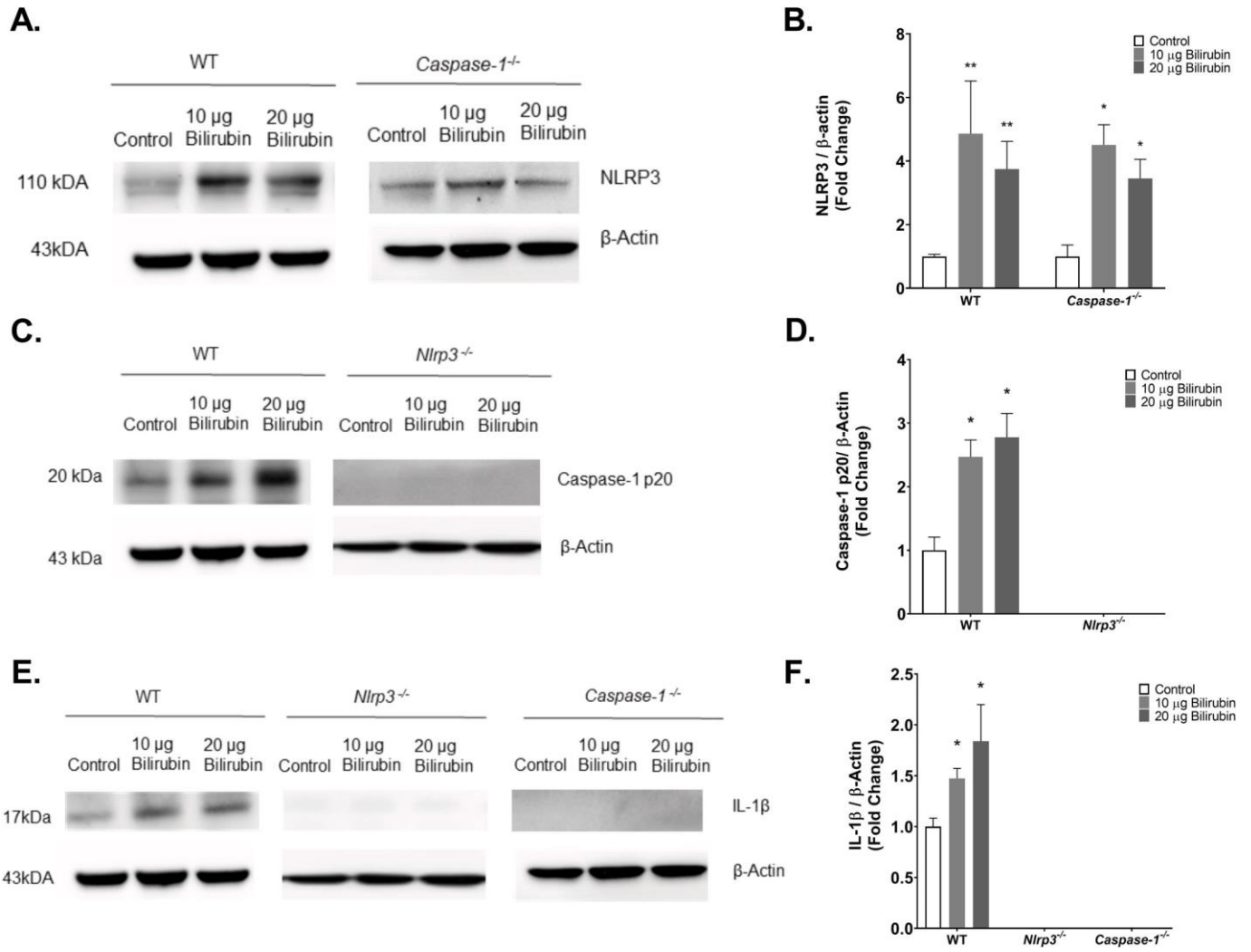

Fig. 4. Bilirubin increases the NLRP3 inflammasome protein level in mouse brain. Immunoblot analysis was carried out in 10 µg and 20 µg bilirubin-injected WT, *Nlrp3*$^{-/-}$, and *Caspase-1*$^{-/-}$ animals. (A, B) NLRP3 protein levels; (C, D) cleaved caspase-1 (p20) and (E, F) IL-1β protein levels indicated that bilirubin treatment results in NLRP3 inflammasome activation. The results are presented as mean ± S.E.M, n = 5. *p < 0.05 and **p < 0.01 compared with untreated control.

4. Discussion

It has been estimated that 20 million babies per year worldwide develop significant jaundice, and hyperbilirubinemia still remains as one of the leading causes of neonatal deaths or long-term neurological impairments. Providing insights into the mechanisms of BIND could serve as the stepping stone toward the reduction of the disease burden (Olusanya et al., 2018). The cytotoxic effects of bilirubin in the central nervous system have been studied extensively, and also the breakdown of nerve cells and mitochondrial membranes, inhibition of glutamate intake, *N*-methyl-D-aspartic acid (NMDA)-mediated toxicity, and intracellular calcium increase have been reported. These events result in cell death with necrosis and apoptosis (Fernandes et al., 2006). In the present study, we demonstrated that bilirubin induces inflammasome activation, and we also revealed the participation of NLRP3 in this activation with *in vivo* and *in vitro* studies.

Many *in vivo* models have been postulated to represent the effects of hyperbilirubinemia in animals. Gunn rats were named based on the discovery of a novel causative mutation for jaundice by Gunn in 1938. Since hyperbilirubinemia reaches its peak level on a PN 16 (Rice and Shapiro, 2008), Gunn rat models could not assist in the discovery of the precise mechanisms of neonatal hyperbilirubinemia. Nguyen and Fujiwara generated a Ugt-1 gene knock-out hyperbilirubinemia mouse model. However, disruption of the Ugt1 locus led to the generation of a lethal mutation, and, therefore, the neonatal Ugt-1 null animals died within two weeks of life. This presented a significant challenge to the research of bilirubin neurotoxicity (Nguyen et al., 2008). In some central nervous system disease studies, *cisterna magna* puncture has been used as a delivery method for therapeutic agents. In 2014, a new kernicterus model was established by injecting bilirubin into the *cisterna magna* using newborn Sprague-Dawley rats (Song et al., 2014). In our study, bilirubin was injected directly into the *cisterna magna* of WT, *Nlrp3*$^{-/-}$, and *Caspase-1*$^{-/-}$ mice. To the best of our knowledge, our group is the first to apply this method to mice. Considering the abundance of knock-out strains in mice compared to rats, we suggest that this model will pave the way for the rational design of research studies toward discovering the molecular mechanisms underlying bilirubin neurotoxicity.

It has been noted that glial cells exhibit varying degrees of susceptibility to bilirubin. (Kumral et al., 2005; Brites, 2011). Previous studies have shown that bilirubin has toxic effects on the morphology, viability, and mitochondrial activity of astrocyte cells. (Amit and Brenner, 1993). The inflammatory effect of bilirubin was also studied in rat primary astrocytes, and the findings of this study suggest that 50 µM bilirubin results in pro-inflammatory cytokine release, including TNF-α, IL-6, and IL-1β through the NF-κB and MAPK pathways (Fernandes et al., 2007). Additionally, it was also shown that 50 µM bilirubin induced pyroptosis

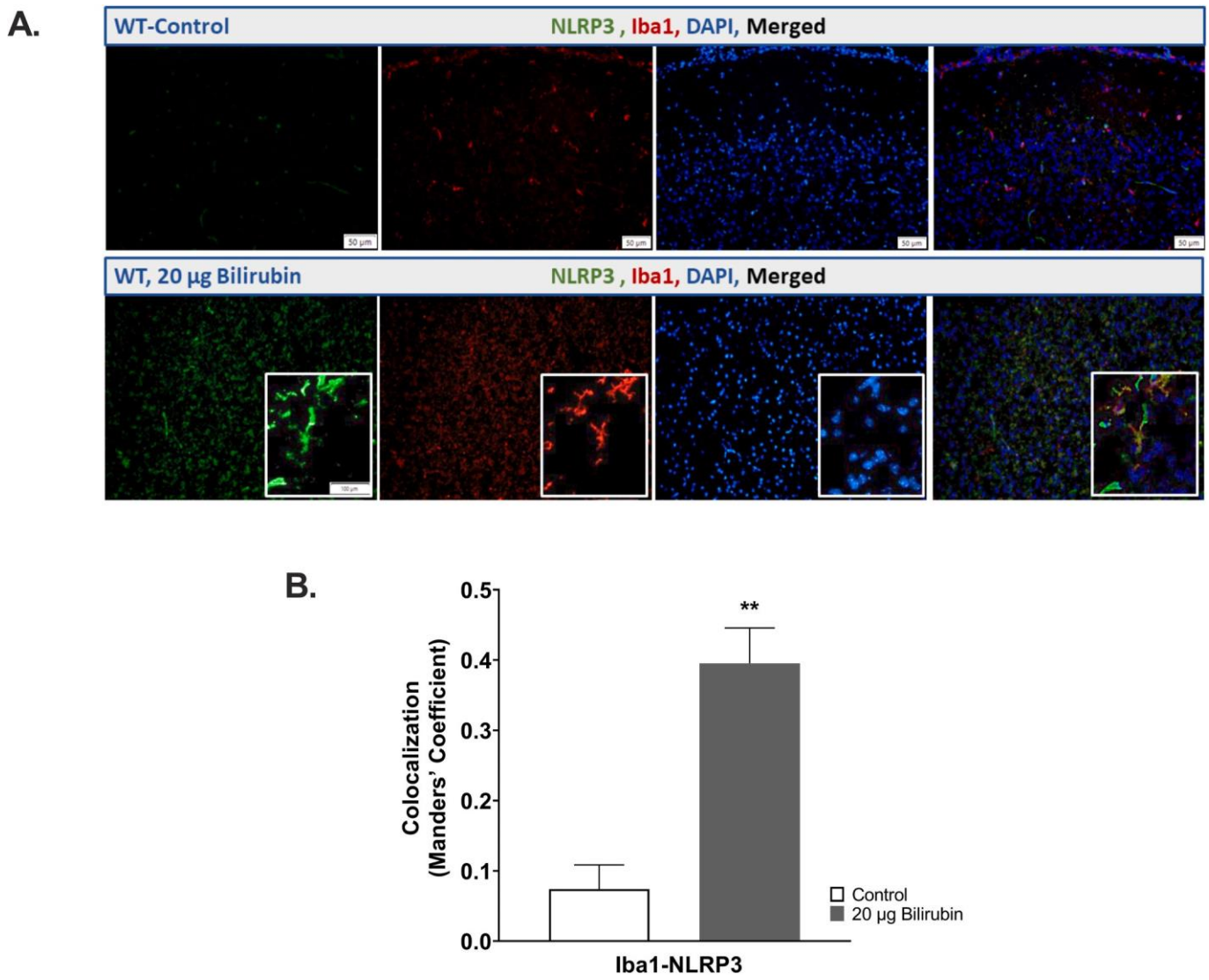

Fig. 5. Bilirubin activated microglial activation and expression of NLRP3. PC sections were stained with the microglia marker Iba1 (red) and NLRP3 (green). Nuclei were stained with DAPI (blue). (A) Increased microglial activation and NLRP3 protein levels were noted. (B) Increased Iba1-NLRP3 co-localization in bilirubin-treated mice are represented in a graph. Data are presented as mean ± S.E.M, n = 5. *p < 0.05 and **p < 0.001 compared to control. Magnification: 10× (A, C); 40× (B).

in rat cortical astrocytes and inhibiting caspase-1 reduced bilirubin-induced cytokine release (Feng et al., 2018).

Microglia are resident macrophages and sensor cells in the central nervous system and have been shown to play an essential role in tissue repair and the regulation of immune and inflammatory activities in the brain (Kreutzberg, 1996). A detailed analysis of sectioned brain tissues with kernicterus revealed the presence of bilirubin pigment within the microglial cells (Gourley, 1997). The initial response of microglia to bilirubin could probably be an increase in phagocytosis, but it has been shown that a 12-h incubation was adequate to trigger apoptosis (Silva et al., 2010). Increased concentration of bilirubin resulted in the induction of microglial activation, as well as inflammatory and phagocytic phenotypes, and subsequent release of the cytokines TNF-α, IL-1β, and IL-6 (Gordo et al., 2006; Silva et al., 2010). In these studies, it has been specified that, in general, the pro-inflammatory status could reach a maximum level within 4 h to 12 h in the presence of 50 μM bilirubin. We have chosen the concentration in our experiments based on published studies (Falcao et al., 2005; Gordo et al., 2006; Silva et al., 2010) and we found that both non-toxic and inflammatory outcomes were obtained following 6 h incubation of N9 microglial cells in 50 μM of bilirubin. We have also tried to evaluate the *in vitro* effects of bilirubin both alone and in combination with LPS. Although the LPS had some impact on the NLRP3 inflammasome, the difference was not as significant as it was with bilirubin alone, so we opted to keep using bilirubin alone.

In our study, we hypothesize that bilirubin toxicity affects NLRP3 inflammasome activation in the brain. We found that 50 μM bilirubin incubation led to the NLRP3 inflammasome priming stage *via* the increase in the NLRP3 and IL-1β expression level in N9 microglial cells. In addition, we demonstrated the increased protein level of NLRP3, cleaved caspase-1 (p20), and cleaved IL-1β in the activation step of the NLRP3 inflammasome. We also confirmed NLRP3 inflammasome activation by bilirubin injection with NLRP3 and caspase-1 staining in CA1 and PC regions of the brain in WT mice. To understand the role of NLRP3 in bilirubin-induced inflammasome activation, NLRP3 was silenced with specific siRNA, and we detected a reduction in the levels of inflammasome parameters *in vitro*. Li and Huang have shown the activation of NLRP3 and caspase-1 in the whole brain of the kernicterus rat model and indicated that bilirubin-induced neurotoxicity could be partially reversed with the inhibition of caspase-1 by VX-765 (Li et al., 2021). In our study, we used NLRP3 and caspase-1 knock-out mice to investigate

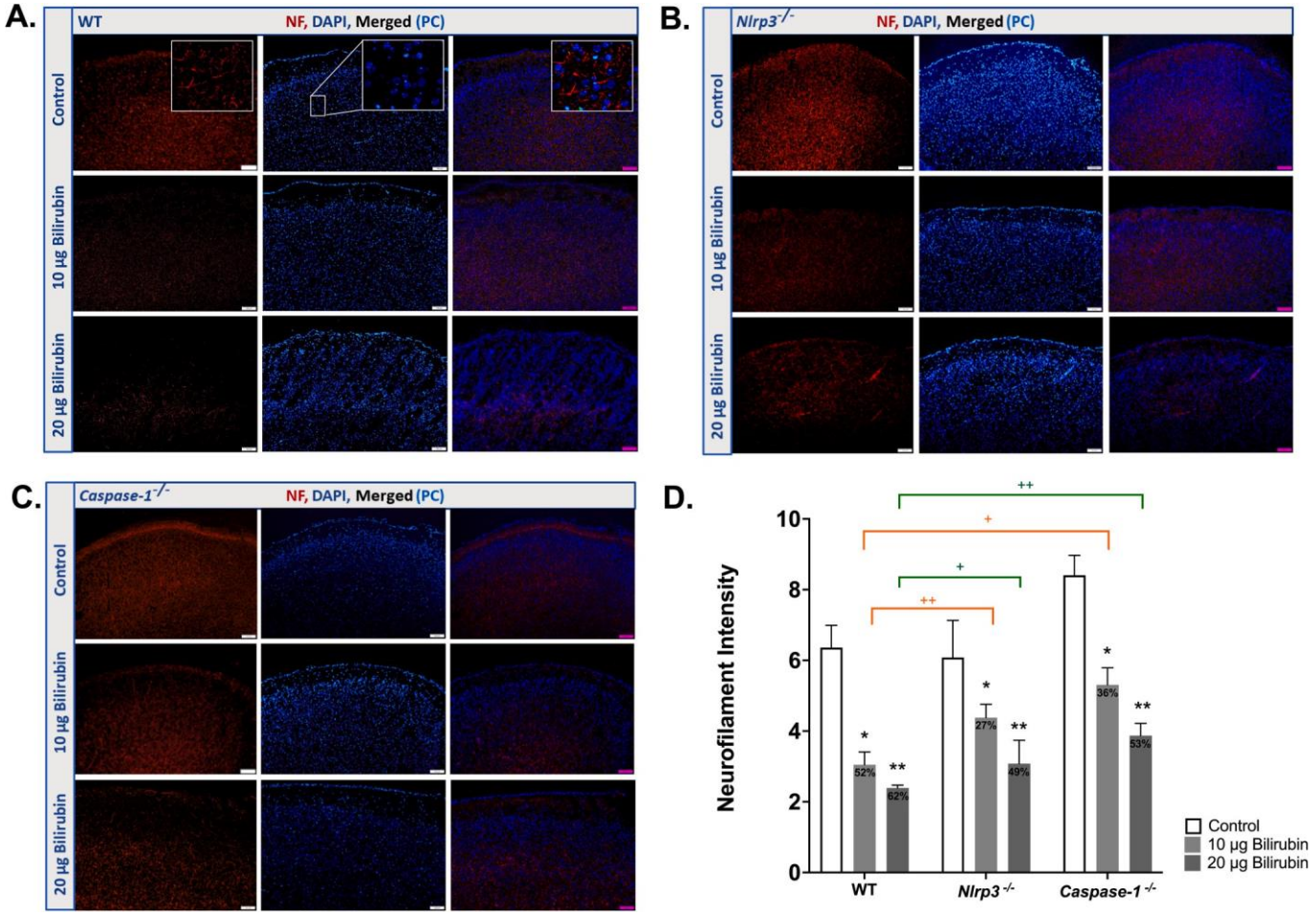

Fig. 6. Bilirubin elicited neuronal damage and decreased expression of NF. PC sections were stained with NF (red); nuclei were stained with DAPI (blue). (A) In WT mice, NF expression was decreased in 10 µg and 20 µg bilirubin groups compared to control. (B) In the *Nlrp3*$^{-/-}$ and (C) *Caspase-1*$^{-/-}$ mice, (D) bilirubin also resulted in a dose-dependent decrease in NF intensity, however less prominent when compared to the WT. Data are presented as mean ± S.E.M, n = 5. *p < 0.05 and **p < 0.01 and compared to control. Statistics of percentage changes of knock-out animals with different doses were presented as + p < 0.05, ++p < 0.01 compared to WT percentage changes. Magnification: 10×.

the interaction between inflammasome and bilirubin toxicity. In the absence of NLRP3 and caspase-1, we determined that the effect of bilirubin could not be the same as in WT mouse brains. Increased NLRP3, IL-1β, and caspase-1 protein levels were demonstrated in WT animals; however, only the NLRP3 protein level was detected in bilirubin-injected *Caspase-1*$^{-/-}$ animals. Because of the absence of IL-1β level in knock-out animals, we can conclude that the NLRP3 complex was not formed in the activation step, but NLRP3 was still expressed in the priming step of the inflammasome. These findings indicate that one of the underlying mechanisms of bilirubin toxicity could be the NLRP3 inflammasome. Li and Huang also indicated that pyroptosis was involved in the pathogenesis of bilirubin neurotoxicity in kernicterus model rats (Li et al., 2021). In our study, we showed that microglial pyroptosis could occur upon bilirubin treatment. We confirmed bilirubin-induced pyroptosis in N9 microglia with PI staining and activation of caspase-1. By co-localization studies in mice, we found that microglia-specific marker Iba-1 levels correlate with NLRP3 in PC regions of bilirubin injected WT mice. Our study supports that the NLRP3 inflammasome could have a role in bilirubin neurotoxicity through microglial activation.

Many studies investigating bilirubin neurotoxicity in newborns have shown that bilirubin mainly causes neuronal damage. The inclination of bilirubin to the cell membrane, especially myelin-rich-membranes, has pointed to neurons as the main targets of bilirubin toxicity (Watchko and Tiribelli, 2013). *In vitro* studies demonstrated that decreased dendritic and axonal branching, reduced neurite extension (Fernandes et al., 2009), as well as attenuation in cell proliferation and increased death by apoptosis (Falcao et al., 2007), are usually observed in neurons exposed to bilirubin(Fernandes et al., 2009). Furthermore, it is well known that, neurotoxicity is frequently accompanied by neuroinflammation (Glass et al., 2010). The accumulation of bilirubin-related neuronal cell death in Ugt1$^{-/-}$ mice was associated with neuroinflammation (Vodret et al., 2017). Microglial cells triggered by bilirubin mediate inflammation-related neuronal cell death through high levels of nitric oxide produced by glutamate uptake (Silva et al., 2012). In our study, like mentioned earlier, we demonstrated that bilirubin administration led to neuronal loss by reducing the number of NF-positive cells. In addition, we showed that NLRP3 inflammasome activation has at least a partial role in bilirubin-induced neuronal loss. In the absence of NLRP3 or caspase-1, the reduction ratio of the total NF-positive cells was not as high as that of the WT animals.

Finally, a number of potential shortfalls need to be considered. First, we employed the N9 murine microglia cell line, which is a well-established and widely used model for investigating various aspects of microglial biology (Stansley et al., 2012). Considering the advantages such as ease of handling and maintenance of cell lines, N9 microglial cell line was preferred for *in vitro* analyses in our study. To support the findings from cell culture, we carried out *in vivo* experiments with WT and knock-out strains. Although we used bilirubin injection to simulate hyperbilirubinemia in those experiments, our findings could be

validated in other models of hyperbilirubinemia, such as Ugt1 knock-out animals (Nguyen et al., 2008) or phenylhydrazine-induced hemolysis model (Rice and Shapiro, 2008). Ultimately, the pathways through which bilirubin exerts its impact on inflammasome activation remain unclear, and therefore warrant further investigation in future studies.

In conclusion, this paper points out a potential relationship between microglial NLRP3 inflammasome activation and bilirubin-induced neurotoxicity. Further elucidation of the mechanisms governing this association could open a new avenue for researchers to propose novel therapeutic strategies for BIND.

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CRediT authorship contribution statement

S.G. and A.K. designed the study. *I.E.* designed and performed the *in vitro* studies. S.C.M., *I.E.*, and S.S. designed and performed *in vivo* studies. S.C.M., *I.E.* and K.U.T. analyzed and interpreted the data. *I.E.* and D.E. wrote the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

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